



**Fluorescence of Bacteria, Pollens, and Naturally  
Occurring Airborne Particles:  
Excitation/Emission Spectra**

**by Steven C. Hill, Michael W. Mayo, and Richard K. Chang**

**ARL-TR-4722**

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## **Fluorescence of Bacteria, Pollens, and Naturally Occurring Airborne Particles: Excitation/Emission Spectra**

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Some of the figures shown here were also shown in *Optics of Biological Particles* (42), but were so reduced in size in that document that it was difficult to read them.

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# 1. Introduction

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## 1.1 Bioaerosols and Other Organic Carbon Aerosols

Airborne biological particles (1–10) are important in the transmission of diseases (11, 12) of humans (e.g., tuberculosis, influenza), farm animals (e.g., coccidioidomycosis, anthrax), agricultural crops (e.g., black stem rust), and trees (e.g., white pine blister rust). Airborne pollens and proteins shed from household pets can cause allergies. Bacteria (e.g., *B. thuringiensis*) used to control certain insect pests are usually disseminated through the air. Bacteria, rickettsia, viruses, protein toxins, and some neurotoxins produced by microbes have been feared as potential airborne biological warfare agents (11–15). The specific types of particles, whether organic, biological, or other, associated with increased mortality and cardiovascular diseases in humans are not presently understood (16). Bioaerosols are a subset of organic carbon aerosols (OCA). Bioaerosols and other OCA can have important effects on climate, through scattering and absorbing light, and acting as cloud condensation nuclei (17–19).

Improved real-time methods for characterizing airborne particles could be useful for a variety of applications, e.g., in monitoring and understanding the spread of diseases of plants, animals, and humans, or in determining the sources of unwanted particles in clean rooms.

In monitoring and studying airborne biological particles, it is important to remember that bioaerosols are a subset OCA.

1. For many types of instrumentation for monitoring certain bioaerosols, the most important interferences and background particles are other bioaerosols and, often to a lesser extent, other OCA.
2. Because of the mixing of biomaterials with nonbiomaterials in the atmosphere, or in soils or water (from which aerosols may be generated), a significant fraction of aerosols are complex mixtures of biological and nonbiological materials. Some measurements of proteins in aerosol particles indicate that around 20% of atmospheric airborne particles contain significant amounts of protein (9, 10), although most measurements of individual aerosols do not look for protein as one of the molecular types.
3. “Nonbiological” OCA is also very important for atmospheric chemistry and physics, and if additional information about OCA in general can be obtained by using some of the instrumentation developed for bioaerosol monitoring, that could be very useful.
4. The distinctions between biological and nonbiological OCA are not so well defined. For example, many nonliving materials generated by biological cells are considered to be biological, at least when they are first separated from their source, e.g., protein toxins or other proteins; lignins, lignans, and ferulic acids from plants; and saxitoxin generated by

dinoflagellates. If a cellulose particle is blown from the surface of a plant it is easy to consider it biological, but if it is blown from the surface of a piece of paper it may be less likely to be termed biological. How much can the biomaterials released by cells be transformed before they are no longer considered biological? For example, terpenoid aerosols that are generated from polymerization and other reactions of volatile terpenes released by plants are not considered biological by at least some researchers. Humic substances are extremely complex materials formed from the breakdown of plant and microbial materials. At what point in the breakdown process are these substances considered “nonbiological”? In biomass burning, what parts of the resulting aerosols might be considered biological? The remaining carbon dioxide is not biological, and the polycyclic aromatic hydrocarbons (PAH) are not considered biological. But how about partially burned proteins where some of the amino acids are still intact?

## **1.2 Some Instrumentation Used to Measure Bioaerosols**

Instruments capable of measuring in real time the laser-induced fluorescence (LIF) of airborne particles (20–44) have been shown to be useful for distinguishing between biological and nonbiological particles. These LIF instruments are the ones most relevant to the present study.

Another type of instrument now used for real-time attempts to partially characterize (determine size distribution and number density) aerosols employs elastic light scattering to count and estimate the size of aerosols. These are known as laser particle counters (LPCs) (45–48). As an aerosol particle traverses an illuminated volume of a LPC, the elastically scattered light is sensed and used to deduce particle size. Alternatively, the aerodynamic particle size is determined from the time it takes a particle to travel a given distance (as measured by light scattering at two positions). Some instruments measure elastic scattering from many angles and try to deduce something about particle shape (49). However, elastic scattering instruments are relatively uninformative regarding chemical composition. Refractive index does depend upon some weighting of the molecules in the particle. However, even when refractive index can be deduced from scattering intensity and patterns, the information content obtainable regarding possible compositions is not great, because an enormous number of molecules/compounds have similar refractive indices.

Mass spectrometry of individual flowing airborne particles is a very promising technique (50–54). However, the instrumentation appears relatively expensive, requiring high vacuums, etc. It tends to be insufficiently robust for routine biodetection monitoring. Also, although the instrumentation appears to have the potential to be extremely informative, with real atmospheric samples, the classification is somewhat less than what might be expected.

An LPC that also measures the LIF of aerosols is commercially available (TSI Inc., Minneapolis) and is able to differentiate among some similarly sized particles composed of different substances, e.g., biological vs. nonbiological materials.



Laser-induced breakdown spectroscopy (LIBS) has been used to measure the atomic emission and plasma emission spectra of atmospheric aerosols (55).

### **1.3 Fluorescence of Biomaterials: Excitation-Emission Spectra**

All biological cells contain fluorescent molecules. The primary fluorescent materials (56) in most bacterial cells are the same: the fluorescent amino acids, tryptophan, tyrosine, and phenylalanine (excitation/emission maxima ~280/350 nm), which are constituents of many proteins; the reduced nicotinamide-adenine dinucleotides (NADH, NADPH) (excitation/emission maxima ~340/450 nm); and the flavin compounds (56–58) (e.g., flavin adenine dinucleotide, flavoproteins, riboflavin) (excitation/emission maxima ~450/520 nm). Even bacterial spores contain significant amounts of flavins and nicotinamide compounds (59, 60). Figure 1 illustrates figuratively the spectra that would be obtained from a mixture of riboflavin, NADH, and protein that is excited with three different excitation wavelengths (450-, 350-, and 280-nm).

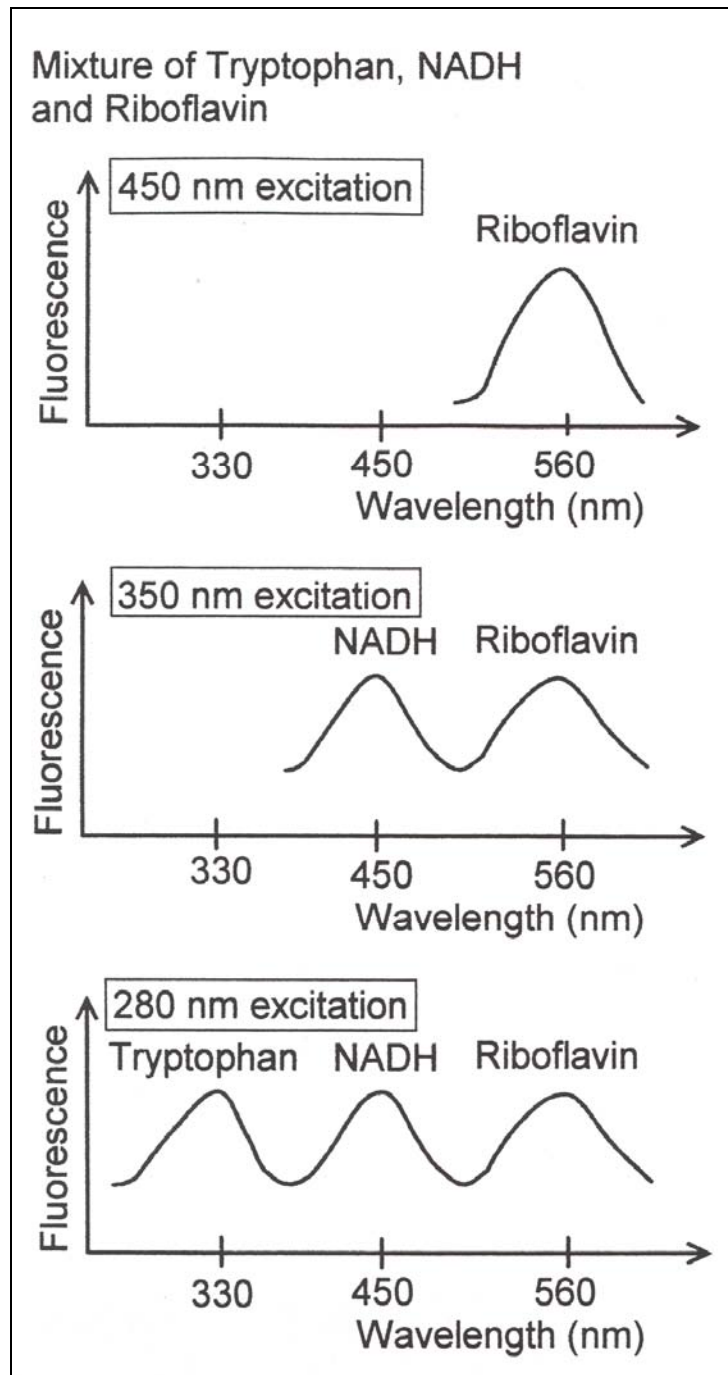


Figure 1. Sketch of the fluorescence from a mixture of riboflavin, NADH, and tryptophan when excited by three different wavelengths.

Note: In the top curve, the 450-nm light excites only the riboflavin; in the middle curve, the 350-nm light excites both the riboflavin and the NADH fluorescence; and in the bottom curve, the 280-nm light has a higher energy per photon absorbed, and this energy is higher than that of the photons emitted from NADH or riboflavin, but the emission is still characteristic of the molecules because the excited electrons decay non-radiatively to their emission band before emission.

Many other fluorescent molecules in biological particles (e.g., chlorophylls, lignins, lignans, ferulic acid) (61– 63) can cause distinct spectral features and thereby increase the differences among fluorescence spectra from various species. Viruses and protein toxins, which typically are not associated with flavins or nicotinamide compounds, commonly contain one or more of the fluorescing amino acids. The spectra of fluorescent molecules depend upon the intracellular environment. For example, the molecular environment of a flavin compound in a spore is different from the environment of the same flavin in a growing cell, because, among other factors, the water content of the spore is low. Dried bioaerosols also contain differing amounts of whatever was dissolved in the liquids in which the particle was suspended (growing or not) or dissolved. Differences in growth media and in the amount of media remaining with the sample particles (because of how the sample was washed, numbers of washes, ionic strength, etc.) can have a large effect on the fluorescence of a sample (25, 27, 29).

Raman emission spectra from biological materials tend to contain more information than fluorescence spectra do. Why not then measure Raman spectra instead of fluorescence? Raman spectra of biological materials are many thousands of times weaker than fluorescence. Typically the single particle spectra of particles drawn through a focal region have good signal-to-noise ratios when the laser intensities are near levels that cause nonlinear effects (in particles around 1- $\mu$ m diameter). Increasing the laser intensity to increase the Raman signal would lead to laser-induced breakdown.

The vibronic absorption by water is a nuisance in using infrared (IR) absorption to distinguish among types of biological particles. A drawback of the fluorescence technique is that the spectra have less distinct features. Nevertheless, the simplicity of the fluorescence spectra can be an advantage in analyzing highly complex mixtures of compounds as are found in biological cells. Autofluorescence excitation and emission (EEM) spectra can help in distinguishing among (64, 65) different species of bacteria. Fluorescence spectra of bacteria (64–69) are broad and tend to be similar for different species. From an instrument design viewpoint, it is much easier to measure the near ultraviolet (UV) and visible fluorescence spectra of individual airborne biological particles flowing through a laser beam than it is to rapidly measure the IR absorption spectrum of an individual particle. Light sources and detectors for exciting and detecting fluorescence are readily available.

The fluorescence spectrum (which may consist of several fluorescence peaks) from a particle or group of particles can help in discriminating among different particle types. In designing autofluorescence-based instruments it is useful to ask: What excitation and emission wavelengths are particularly useful for discriminating (1) between biological and nonbiological aerosol particles, and (2) among various types of biological aerosols? EEM spectra such as those presented here can help address these questions.

Two criteria for an appropriate excitation wavelength are as follows:

1. At the appropriate excitation wavelengths, the resulting fluorescence spectra contain information for discriminating among types of biological particles, and between biological and nonbiological particles. The fluorescence spectra should have the least interfering fluorescence from nonbiological particles. The wavelength should be short enough to generate informative spectra, but not short enough to unnecessarily generate fluorescence from potential interferents (e.g., clay minerals). Since the absolute magnitudes of fluorescence are difficult to measure, having multiple fluorophores with different peak emissions offers the possibility of using the ratios between fluorescent regions in discrimination.
2. At the appropriate excitation wavelengths, measurable fluorescence signals can be obtained from small particles with relatively inexpensive and compact lasers.

In trying to develop improved autofluorescence-based bioaerosol sensing instruments, we have been hindered by a lack of published EEM data for biological compounds, bacteria, pollens, spores, and nonbiological particulates.

#### **1.4 Overview**

In this report we present measured fluorescence EEM spectra of several types of biological materials, including bacteria, pollens, spores, and grass leaves; some inorganic particles; and an oil. We use these EEM spectra to present our views of useful wavelengths for autofluorescence-based instruments which monitor airborne particles. In considering the design of fluorescence based detectors, we have emphasized readily available, inexpensive laser sources, ones which might be useful in commercial biodetection systems. It is useful to have a set of EEM spectra taken with the same instrument and over the same wavelength ranges, so that one need not wonder if differences between spectra might be artifacts attributable to different instruments or techniques. Most of the EEM spectra shown here were normalized by the intensity of the incident light so that comparisons between samples can be made. The normalized EEM spectra from many samples should be useful for designing future autofluorescence-based biological detection systems.

In section 2 we describe the samples we investigated and the spectrofluorometer we used to measure EEM spectra. In section 3 we present EEM spectra, and in section 4 we discuss those spectra. In section 5 we discuss, with the EEM spectra presented in section 3, wavelengths and lasers that should be particularly useful for autofluorescence-based instruments that detect and discriminate among biological materials. We summarize the findings in section 6.

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## 2. Experimental

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### 2.1 Samples

NADH, riboflavin, tryptophan, *B. subtilis* (vegetative cells) ATCC 6633, *Pseudomonas fluorescens*, *Micrococcus lysodeikticus*, *Staphylococcus aureus*, and *Clostridium perfringens*, were obtained from Sigma Chemical Co. *B. anthracis* (Vollum) vegetative cells and spores were obtained from Dugway Proving Ground, Dugway, UT. *Yersinia pestis* was obtained from Ft. Detrick, MD. The pollens (pecan, paper mulberry, corn, ragweed) were obtained from Polysciences Inc., Warrington, PA. Glass beads were obtained from Peirce Chemical Co., Rockford, IL. Kaolin particles were obtained from Particle Information Services, Grants Pass, OR. The sample of lawn grass shown was freshly picked.

The samples listed as “wet” were suspended in deionized water at a stock solution concentration of 1 mg/ml. The samples were vortexed and pipetted vigorously to disperse aggregates. Stock bacteria solutions were diluted to a final concentration of 0.1 mg/ml. Solutions were prepared from frozen lyophilized cell samples. Fluorescence spectra were routinely obtained within 30 min of preparation. Riboflavin and NADH were diluted to a final concentration of 1  $\mu$ g/ml. *B. anthracis* spores (Ames, Sterne, and Vollum strains) were measured in quartz cuvettes at a concentration of  $10^7$  Colony Forming Units (CFU) per ml in deionized water.

### 2.2 Fluorescence Measurements

The fluorescence emission spectra of each sample was measured using a Fluorolog-2 Model FL2T2 Spectrofluorometer System (SPEX Industries, Inc., Edison, NJ), as illustrated schematically in figure 2. This continuous wave spectrofluorometer consists of a T-box sampling module with a 90°/22.5° selection mirror to reflect the fluorescence from either the front-face or right angle of the sample (either in the 22.5° reflection mode for dry sample, or the 90° collection mode for wet samples). Double-grating (1200 gr/mm) spectrometers are used for both the excitation and emission in order to increase the stray and scatter light rejection and spatial resolution. All the spectra of dry samples presented were acquired using the ratio mode (sample/reference=s/r).

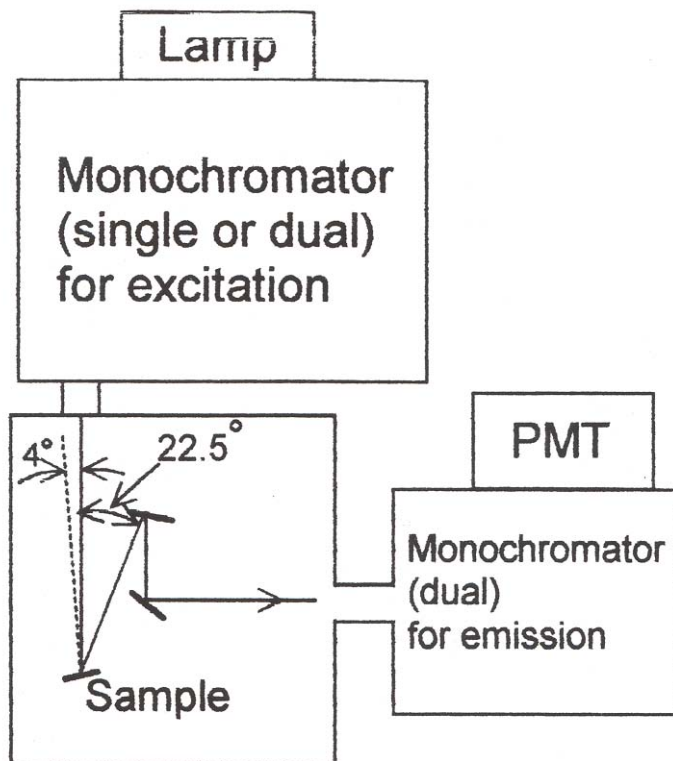


Figure 2. Schematic of the T-cell spectrophotometer.

The excitation and emission spectrometer slits were set for 1 nm bandpass for all measurements in order to reduce effects of scattered light on the spectra. EEM fluorescence spectra were acquired by varying the excitation wavelength from 250 nm to 530 nm at 20 nm bandpass increments. The emission was measured starting at 20 nm above the excitation wavelength. The ending emission wavelength was 20 nm before the second order wavelength line, or 800 nm, whichever was smaller. The integration time was 0.1 s for all samples. No background correction or data smoothing was used. The excitation and emission wavelengths were varied automatically.

The sample suspensions were placed in quartz cuvettes (1 cm) in the T-box sampling module. The quartz cuvettes were excited at normal incidence and the fluorescence emission measured at 90°.

Solid samples were measured as follows. Bacteria, pollen, and the biological compounds were pressed into a solid sample holder (SPEX Model 1933) in the T-box sampling module without a cover slip. The solid sample holder does not exhibit any significant fluorescence. The dry sample is then illuminated with light from the 450 W xenon lamp through the double-grating monochromator at an angle of about 4° from normal incidence. Fluorescence emission is measured through the front face port of the T-box sampling module, which is at 22.5°. Therefore, the total angle between the normal to the sample and the emission measurement is

approximately  $4^\circ + 22.5^\circ = 26.5^\circ$ . The sample was rotated away from the emission port to reduce the noise from specular reflection and scattered light. Leaf samples were secured in the solid sample holder and illuminated with the excitation wavelength at normal incidence. Fluorescence emission was measured at  $22.5^\circ$ . To help validate that scattering of the lamp light did not have a significant impact on the fluorescence spectra, we also illuminated some of the samples with the 488 nm emission from an argon ion laser, which was passed through a linepass filter before illumination. The spectra obtained (not shown) with the lamp excitation set to 488 nm were similar to those obtained with single wavelength excitation from the laser.

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### **3. EEM Spectra**

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We first present spectra of some common fluorescent biological molecules (tryptophan, NADH, and riboflavin), which help in understanding the spectra that follow. We then present spectra of bacteria, spores, pollens, and some leaves of grass. Last we show spectra of selected nonbiological particles: glass beads and kaolin, which have a weak fluorescence; iron oxide and iron particles, which have negligible fluorescence; and fog oil, which has a strong fluorescence. For the biological molecules, cells, and spores, we typically obtained some spectra of both dry and wet samples. The spectra depend upon the degree of hydration, and upon the concentrations of the molecules. Multiple absorption and re-emission in highly concentrated (e.g., solid) samples can shift spectra to longer wavelengths. The water content of airborne particles depends upon the humidity, the particle size, and the way the particle was formed.

#### **3.1 Biological Compounds and Fresh Leaves**

Figure 3 shows the EEM spectra of dry NADH. The peak emission occurs near 450 nm, and the peak excitation is at 380 nm. The NADH is excited well by energy at the shortest wavelengths used. Even at 250 nm excitation, the peak fluorescence intensity is approximately 2/3 the peak emission intensity.

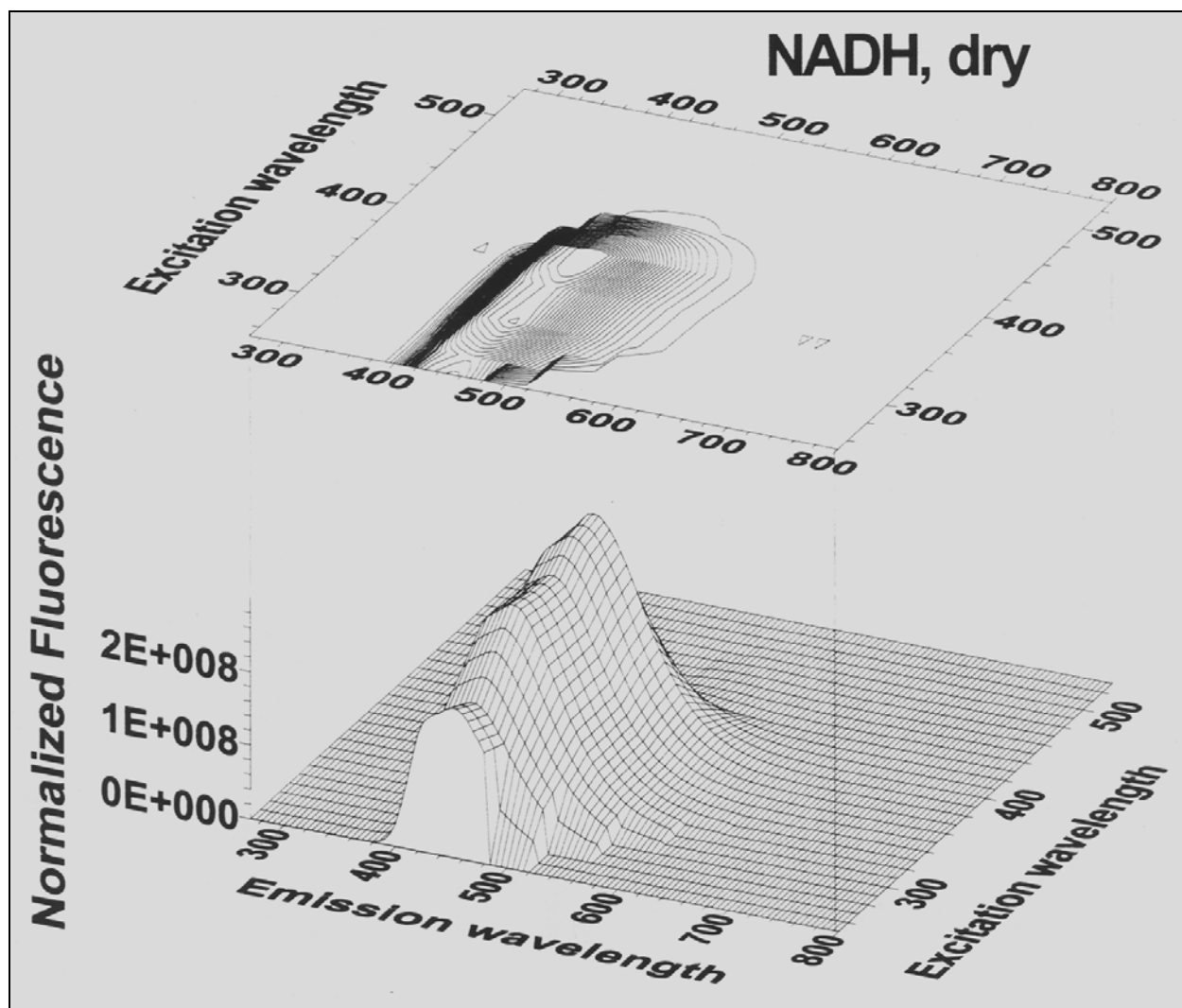


Figure 3. EM spectra of dry NADH.

Note: The signal was corrected for the variation in excitation lamp intensity. The spectrometer slits for excitation and emission were set for 1-nm bandpass. The excitation wavelength was varied from 250 nm to 530 nm with 20-nm bandpass increments. The emission was measured starting at 20 nm above the excitation wavelength. The ending emission wavelength was 20 nm before the second-order wavelength line, or 800 nm, whichever was smaller. The integration time was 01 s for all samples. No background correction or data smoothing was used.

Figures 4 and 5 presents the EEM spectra of riboflavin, dry and wet, respectively. The peak emission from riboflavin in water is near 520 nm. The peak emission from dry riboflavin, on the other hand, is shifted approximately 45 nm to the red, near 565 nm. We have also measured the EEM spectra of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in water, and found the spectra to be similar to the spectrum of riboflavin in water.



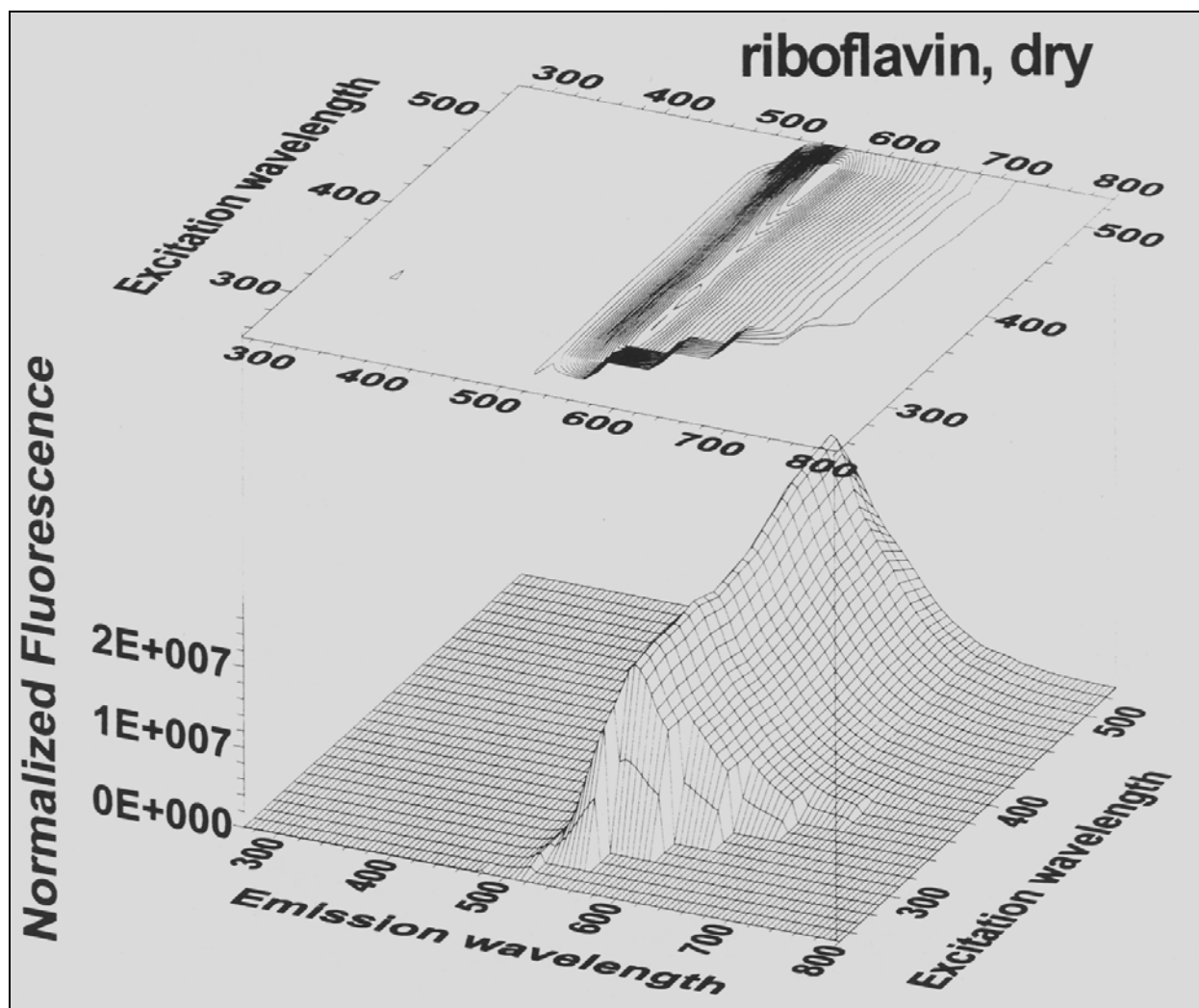


Figure 4. EEM spectra riboflavin, dry.

Note: Other parameters are as in the note with figure 3 and in the text.

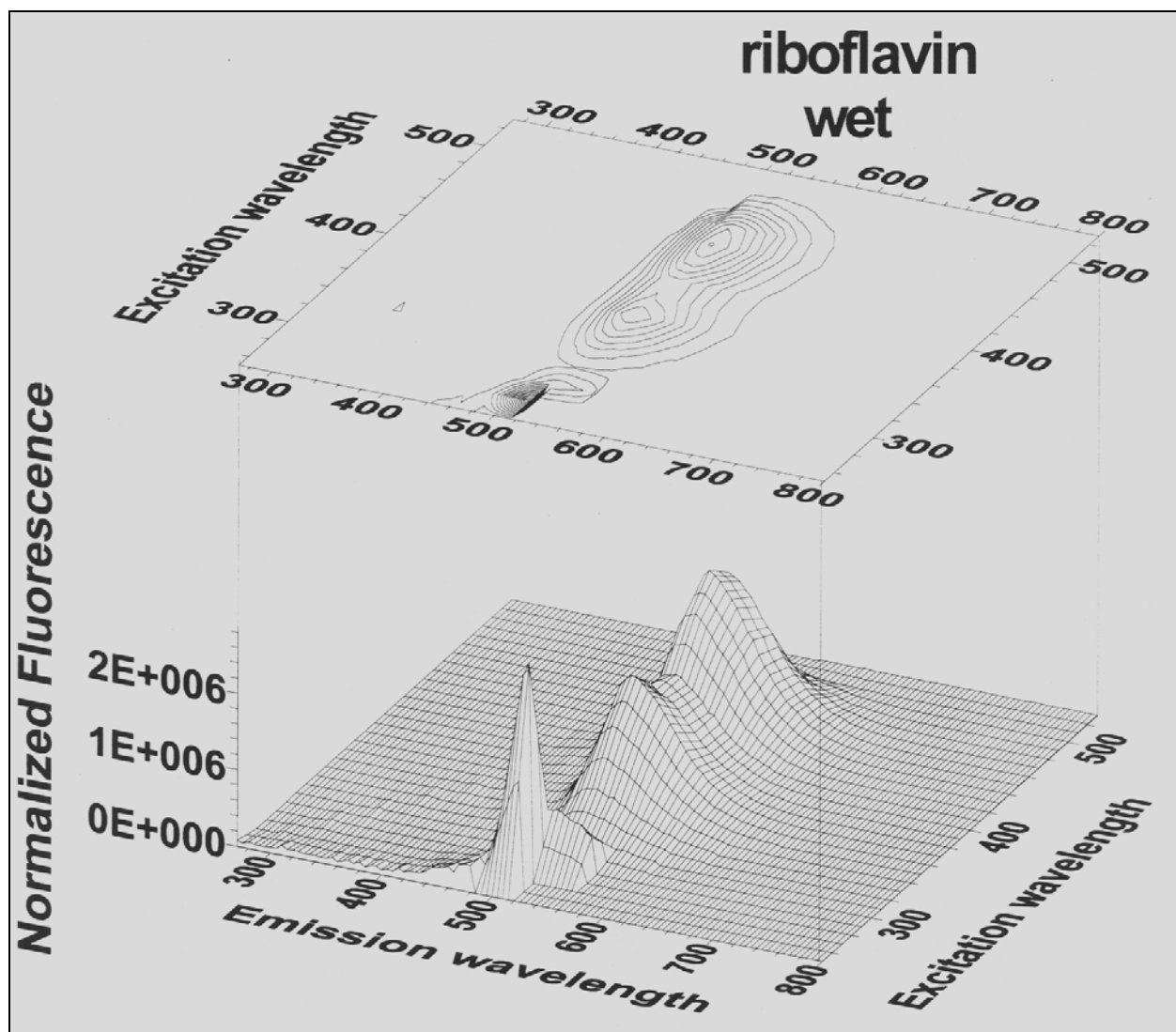


Figure 5. EEM spectra riboflavin, wet.

Note: Other parameters are as in figure 3 and in the text.

Figure 6 and 7 show the EEM spectra of tryptophan dry and wet, respectively. The emission from dry tryptophan was so large that at its peak it saturated our detector. We measured the data with the peak saturated (instead of decreasing slit widths for this sample) because, for comparisons among samples it is useful to have the spectra with the same slits widths. The reason the EEM spectrum of the tryptophan in water decreases more rapidly toward the lowest excitation wavelengths (270 and 250 nm) is that the sample was not corrected for the reference beam.

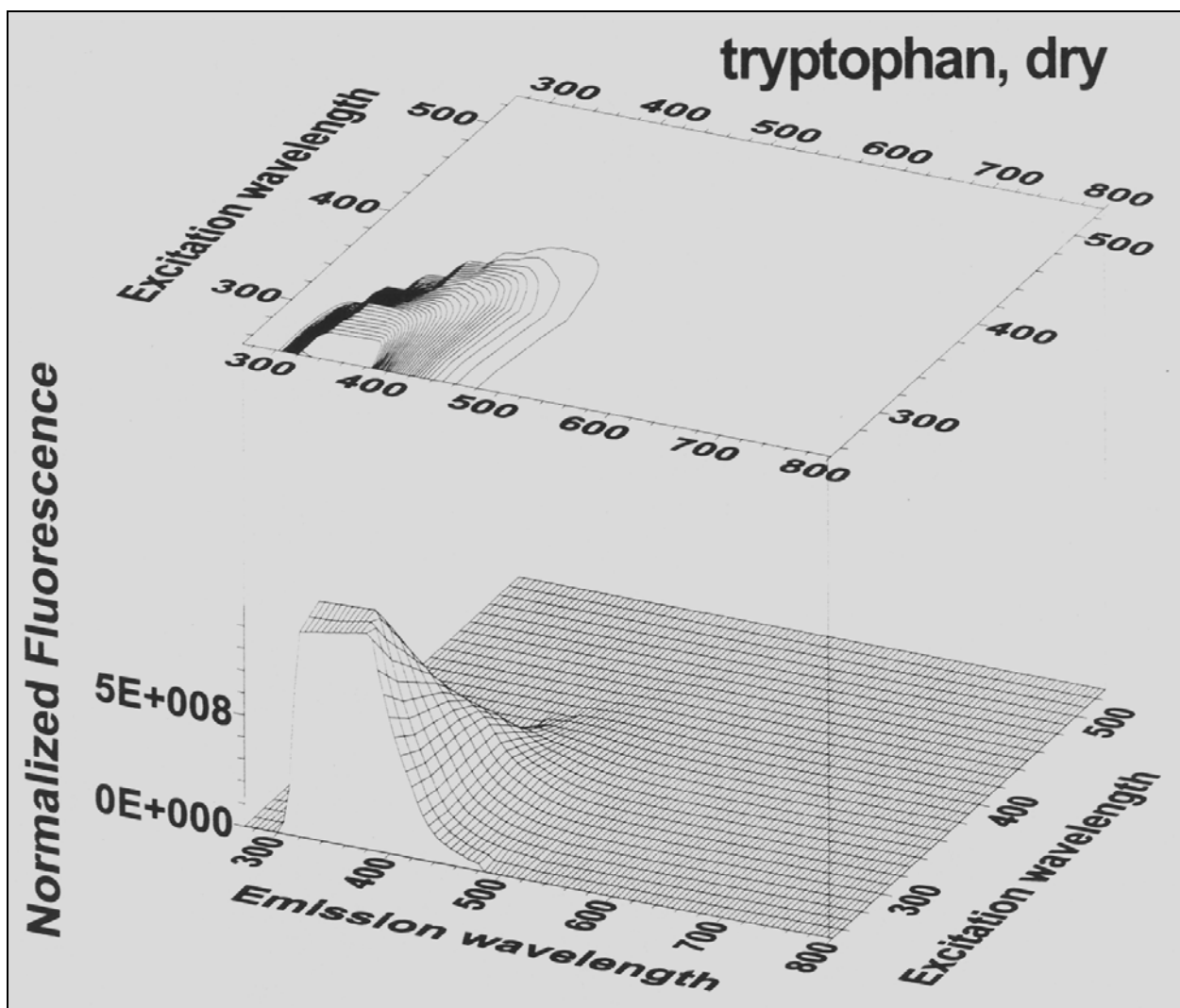


Figure 6. EEM spectra of tryptophan, dry.

Note: Other parameters are as in figure 3 except that (b) was not corrected for the lamp intensity.

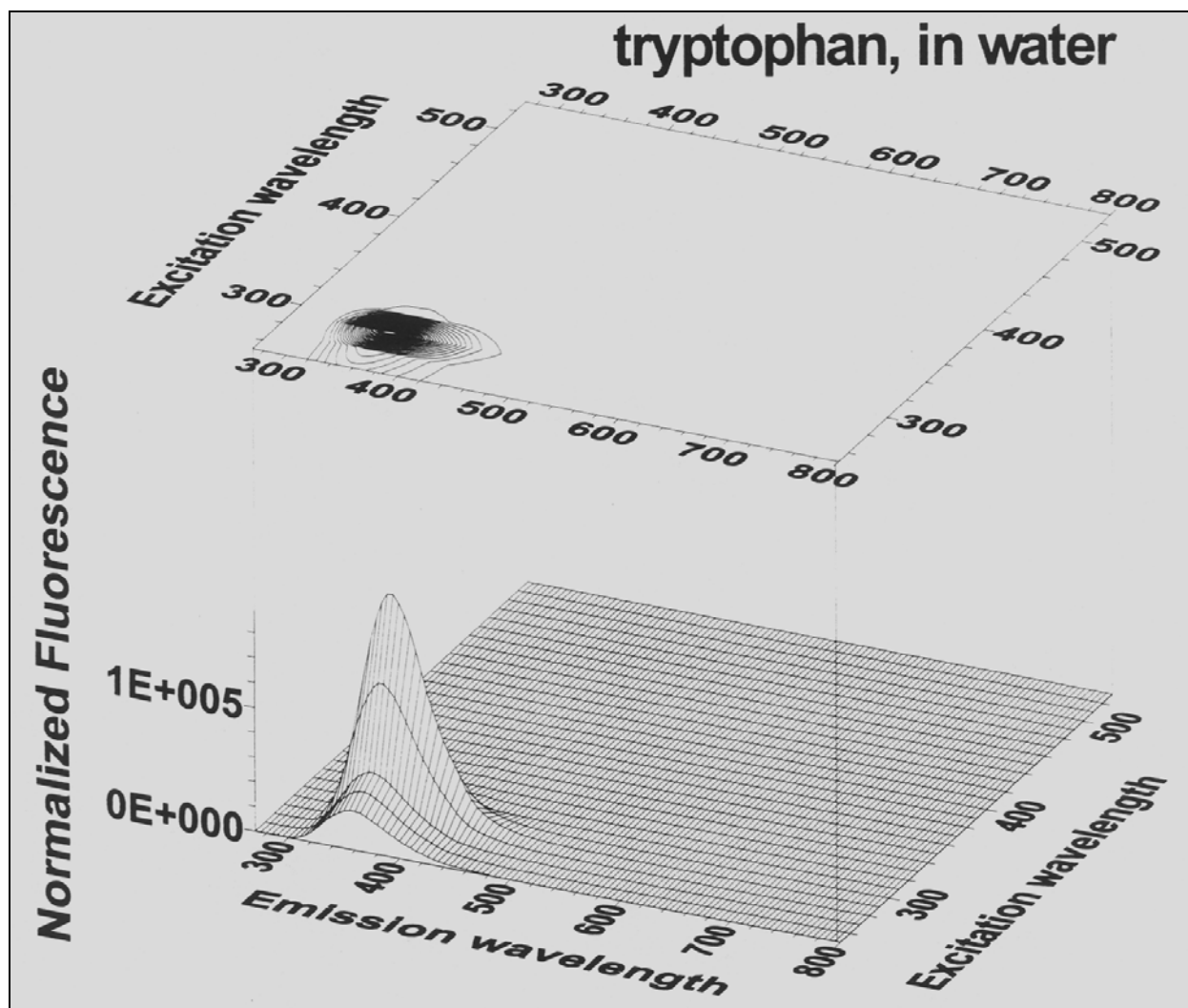


Figure 7. EEM spectra of tryptophan, wet.

Note: Other parameters are as in figure 3.

The EEM spectra of tyrosine in water are illustrated in figure 8. Note how the amplitude is lower than that of tryptophan.

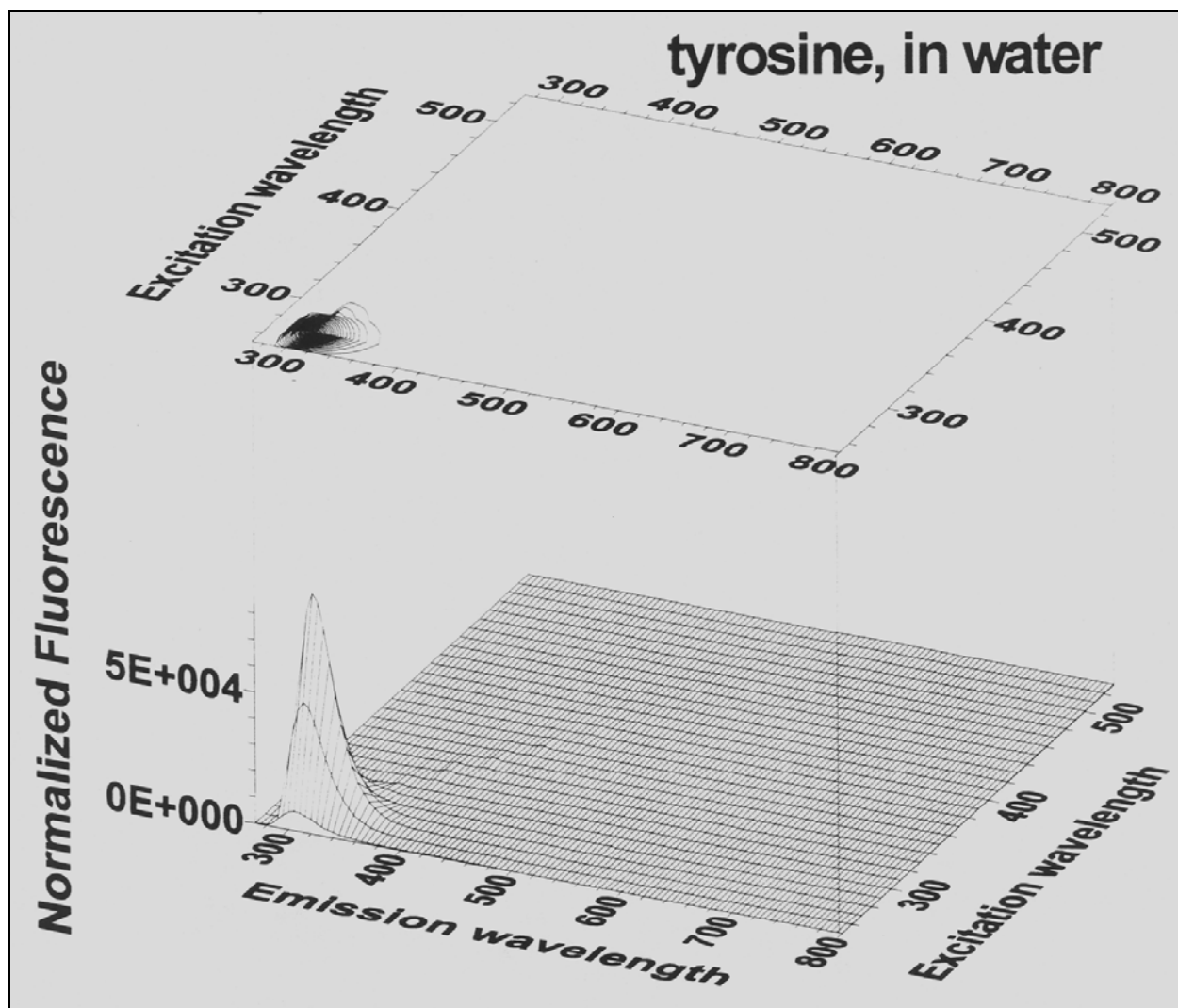


Figure 8. EEM spectra of tyrosine in water.

Note: Other parameters are as in figure 3 except that the spectra were not corrected for the lamp intensity.

The EEM spectra of fresh grass leaves are illustrated in figure 9. A reason to show this spectrum is to demonstrate the spectra of chlorophyll. Some airborne particles, e.g., particles made from droplets that were generated from waves on oceans, ponds, or rivers, may contain chlorophylls from algae or other plants. Bacterial chlorophylls tend to emit further toward the infrared than does the leaf chlorophyll shown here (which has a peak emission near 685 nm). The peak excitation wavelengths in the chlorophyll region shown here are between 430 and 490 nm. We have also measured (not shown) spectra of other plants (e.g., beech leaves, pear leaves), which exhibit peaks both in the chlorophyll region and in the region where flavins fluoresce. The spectra we have measured for moss (not shown) have a very weak fluorescence, which is dominated by chlorophyll, but which does not exhibit measurable fluorescence in the region where NADH or flavins would emit.

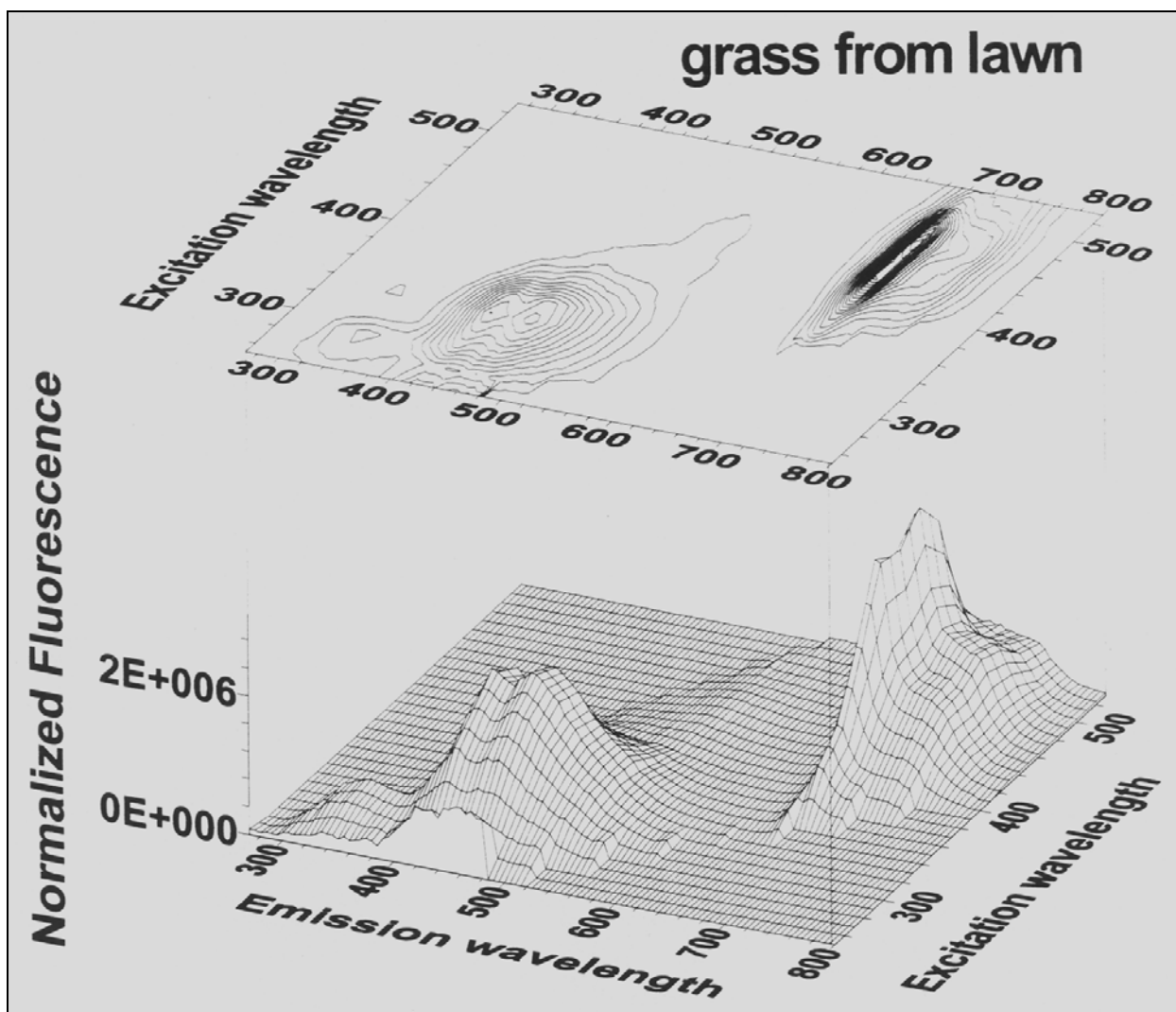


Figure 9. EEM spectra of fresh grass leaves.

Note: Other parameters are as in figure 3.

### 3.2 Bacterial Cells

Figures 10 and 11 show the EEM spectra of *B. subtilis* vegetative cells, dry and as a suspension in water, respectively. The excitation efficiency of the dry sample decreases rapidly as the excitation wavelength decreases below 290 nm (the spectra were corrected for the variations in the lamp intensity). The data in figure 11 was not corrected for the lamp intensity.

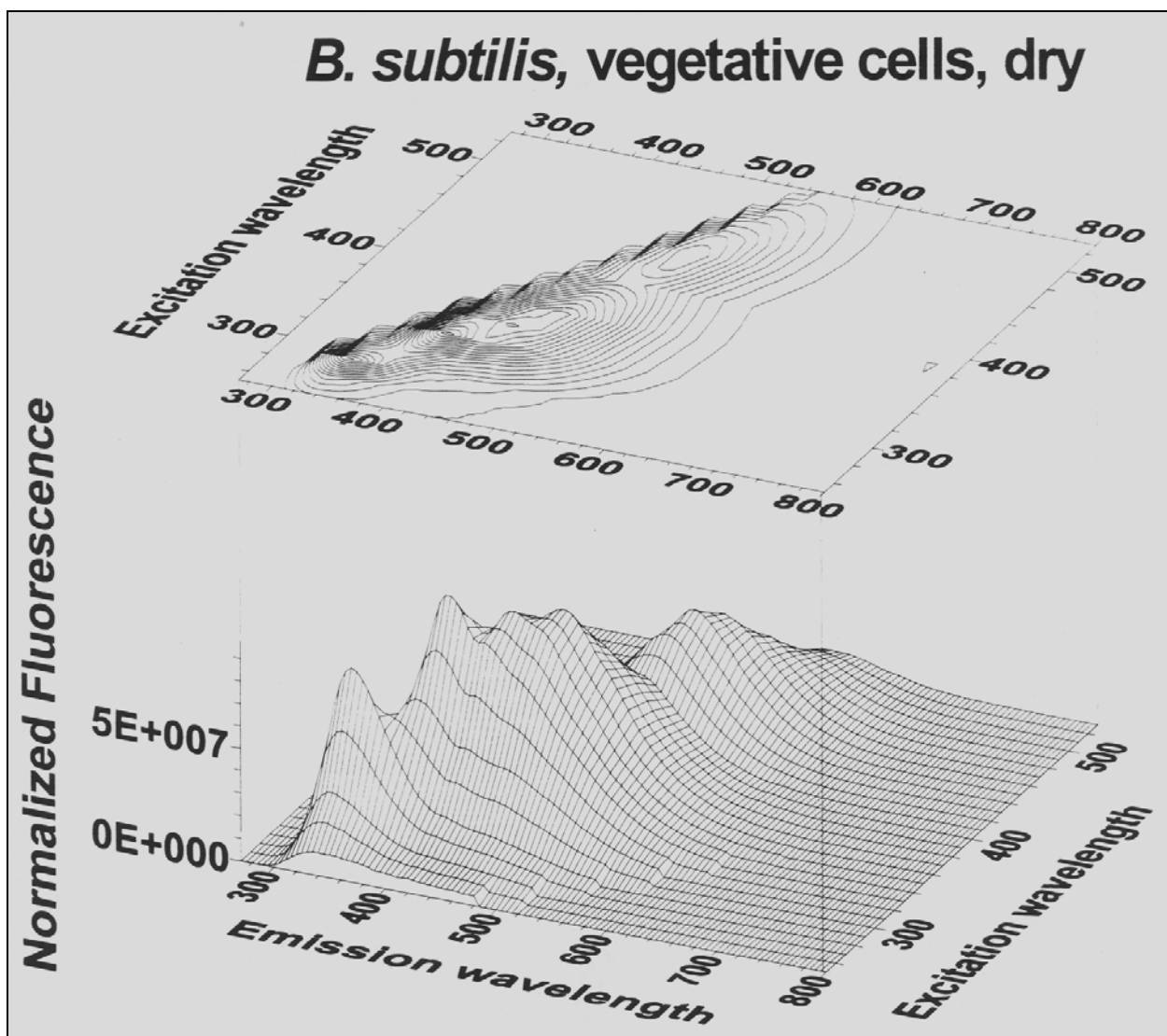


Figure 10. EEM spectra of *B. subtilis* vegetative cells, dry.

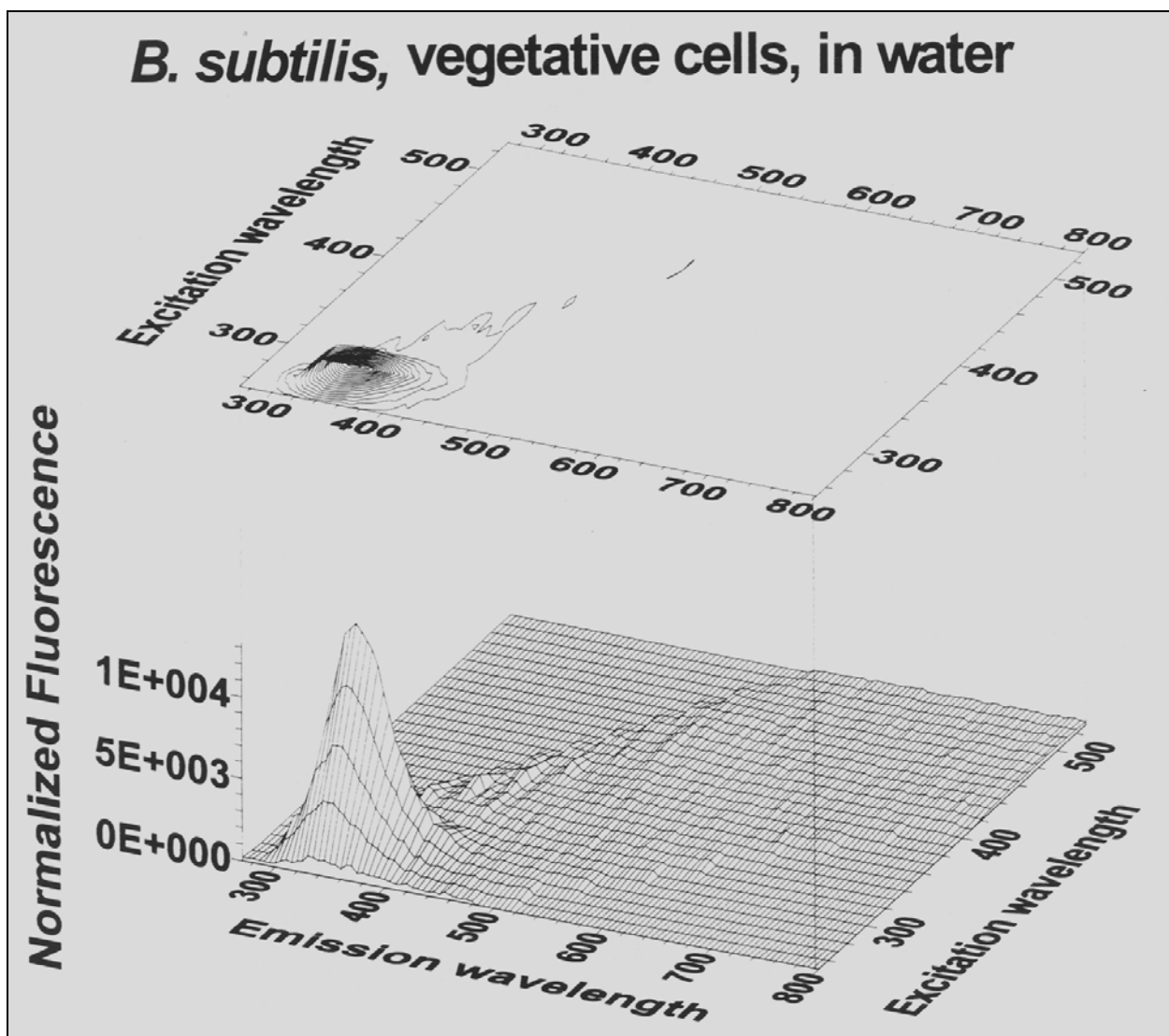


Figure. 11. EEM spectra of *B. subtilis* vegetative cells, in water.

Figures 12 and 13 show EEM spectra of *B. anthracis* (Vollum), showing vegetative cells in figure 12 and spores in figure 13. The bacteria were suspended in water in each case.



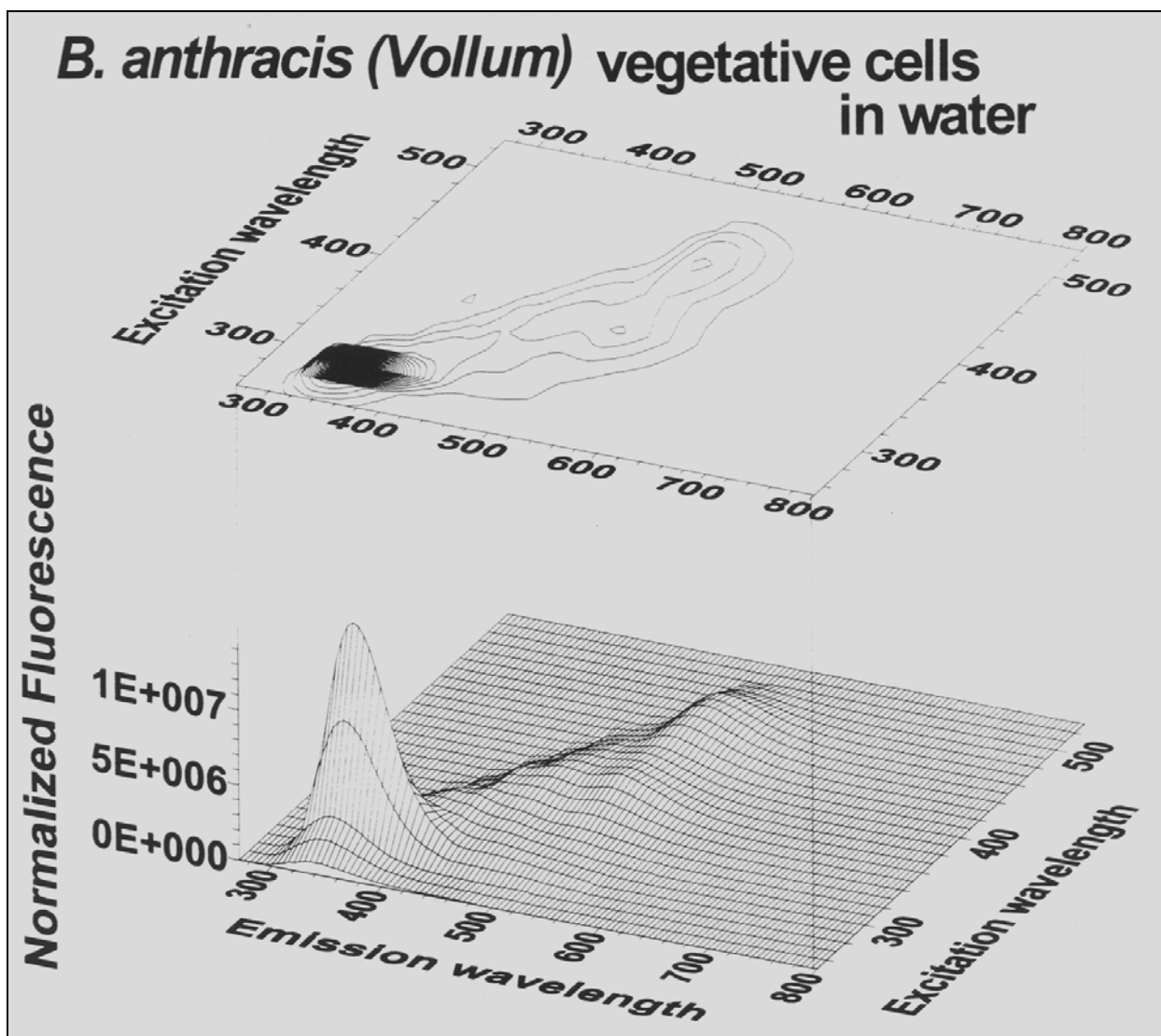


Figure 12. EEM spectra of *B. anthracis* (Vollum) vegetative cells in water, corrected for the lamp intensity.

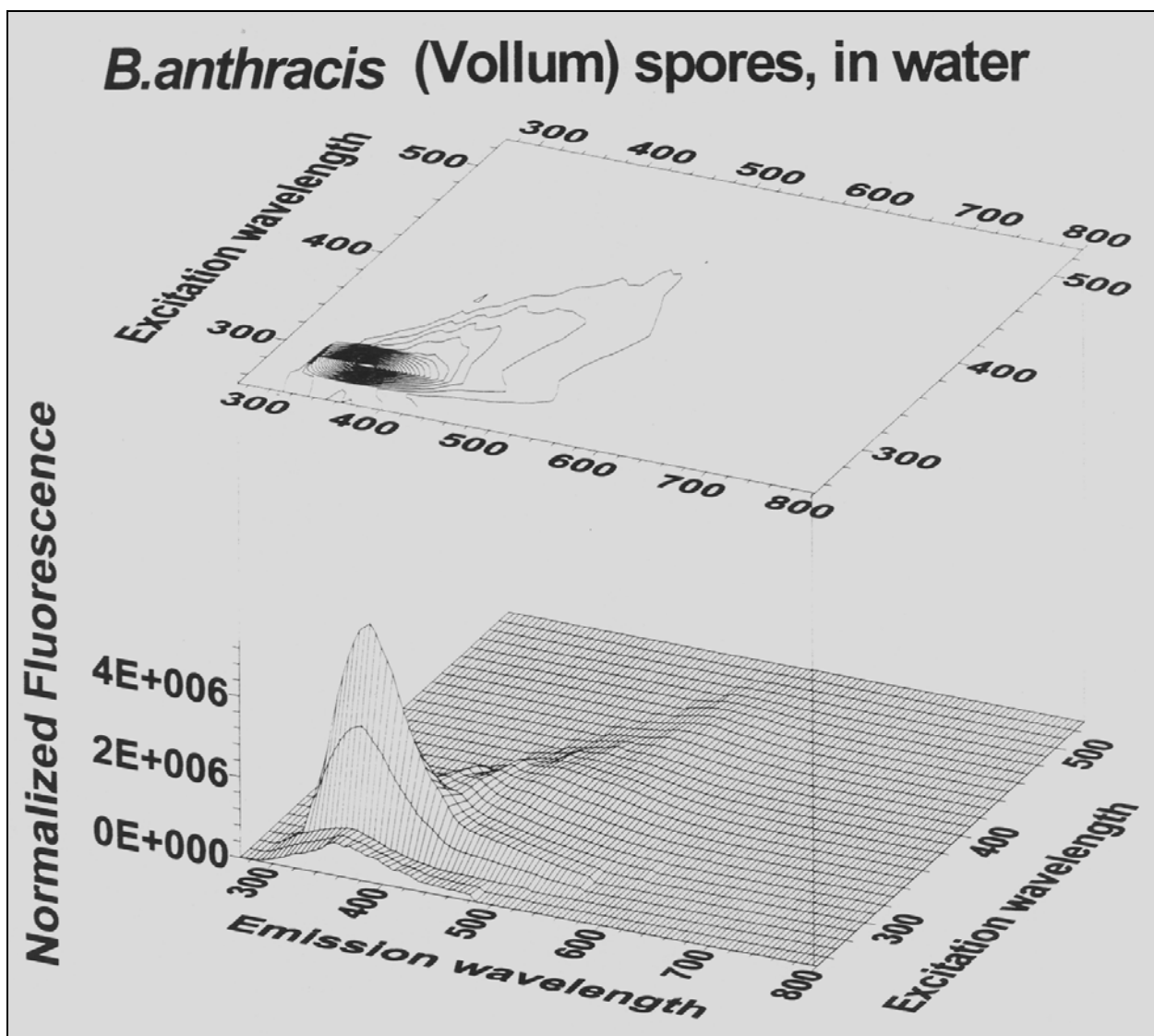


Figure 13. EEM spectra of *B. anthracis* (Vollum) spores in water, corrected for the lamp intensity.

Figures 14–21 present the EEM spectra of *Pseudomonas fluorescens* (dry), *Yersinia pestis* (dry and in water), *Micrococcus lysodeikticus* (dry and in water), *Staphylococcus aureus* (dry), and *Clostridium perfringens* (dry and in water), respectively. The spectra were corrected for the lamp intensity, except for *M. lysodeikticus* in water and *C. perfringens* in water.

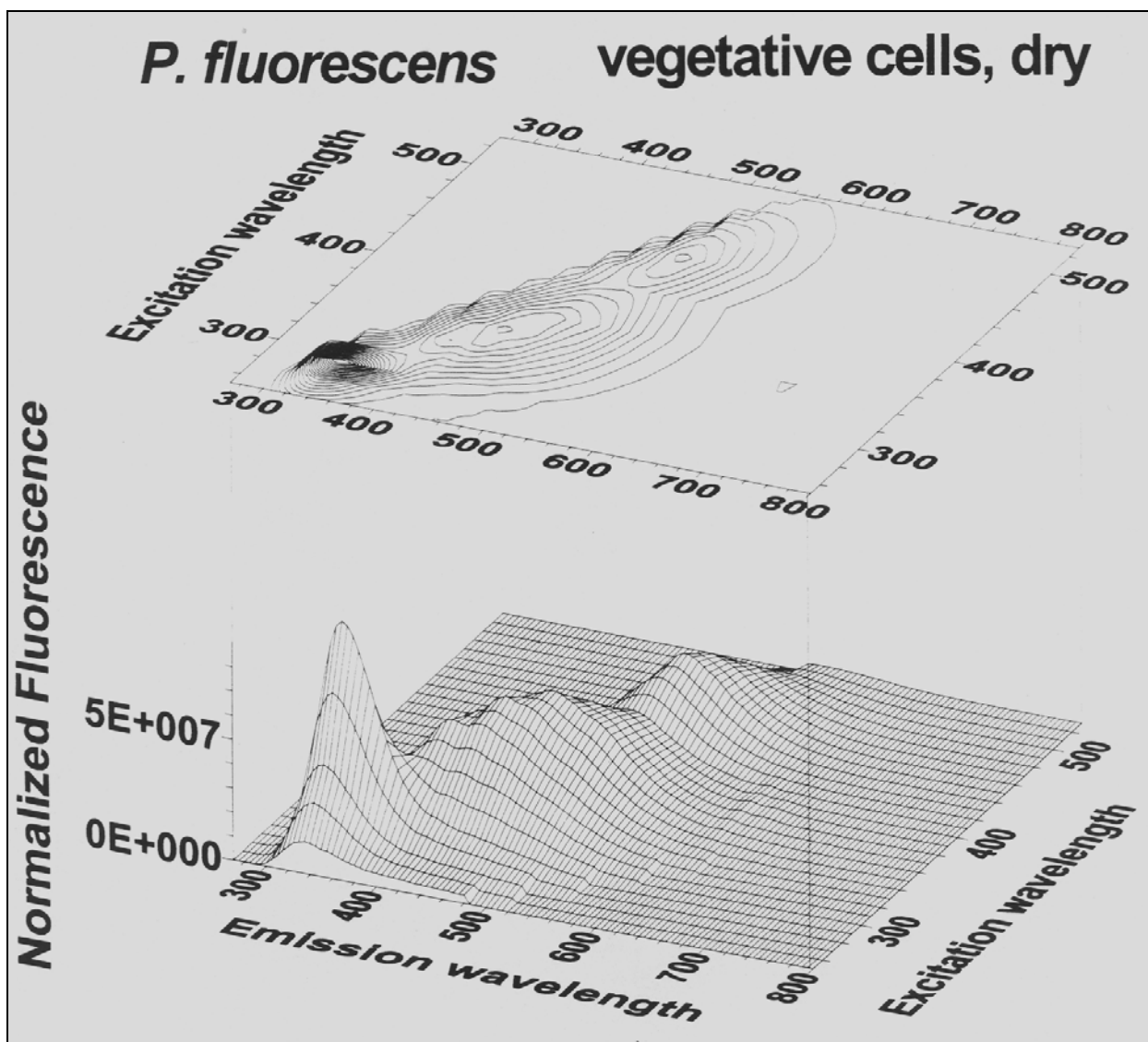


Figure 14. EEM spectra of *Pseudomonas fluorescens* vegetative cells, dry.

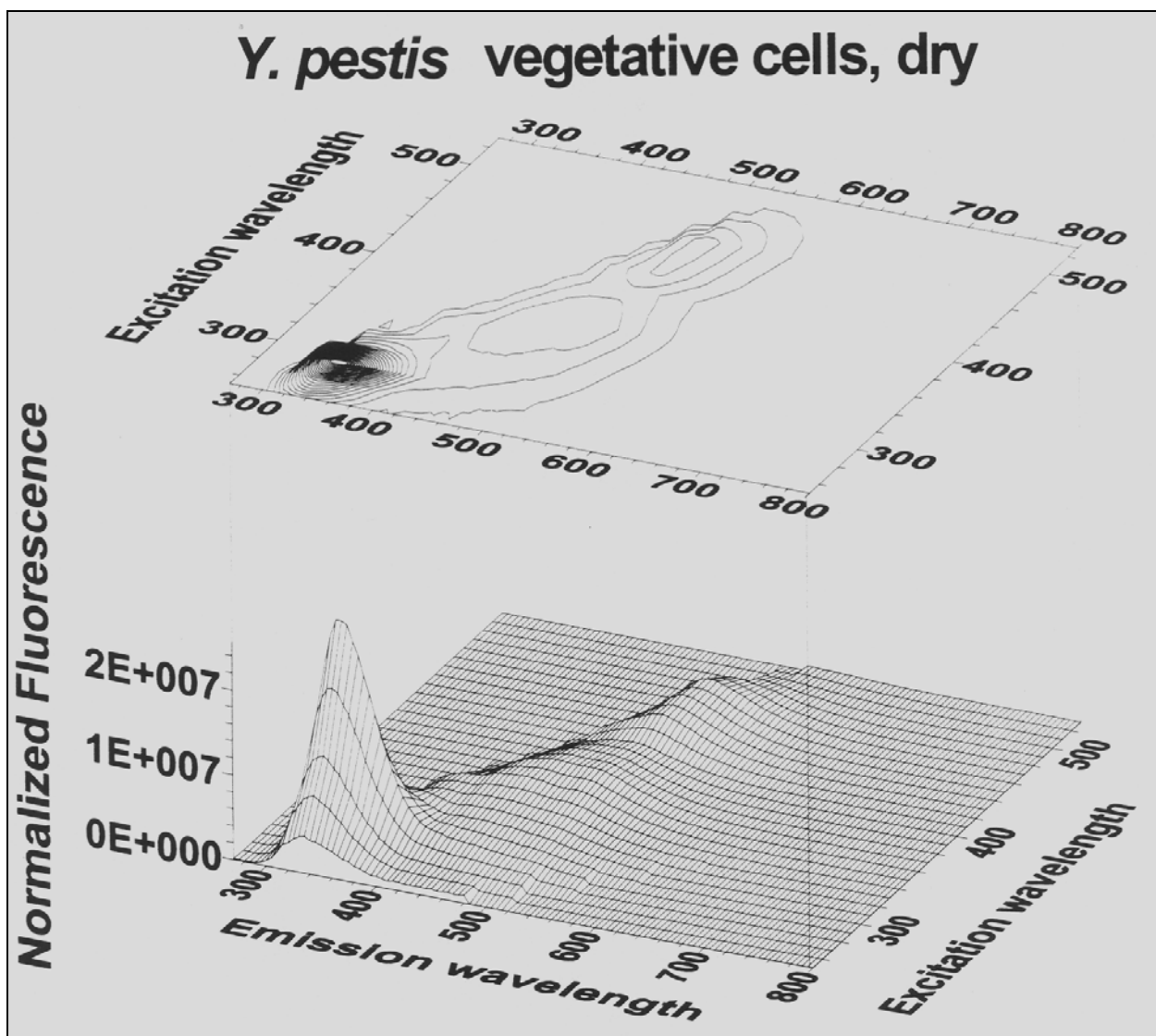


Figure 15. EEM spectra of *Yersinia pestis* vegetative cells, dry.

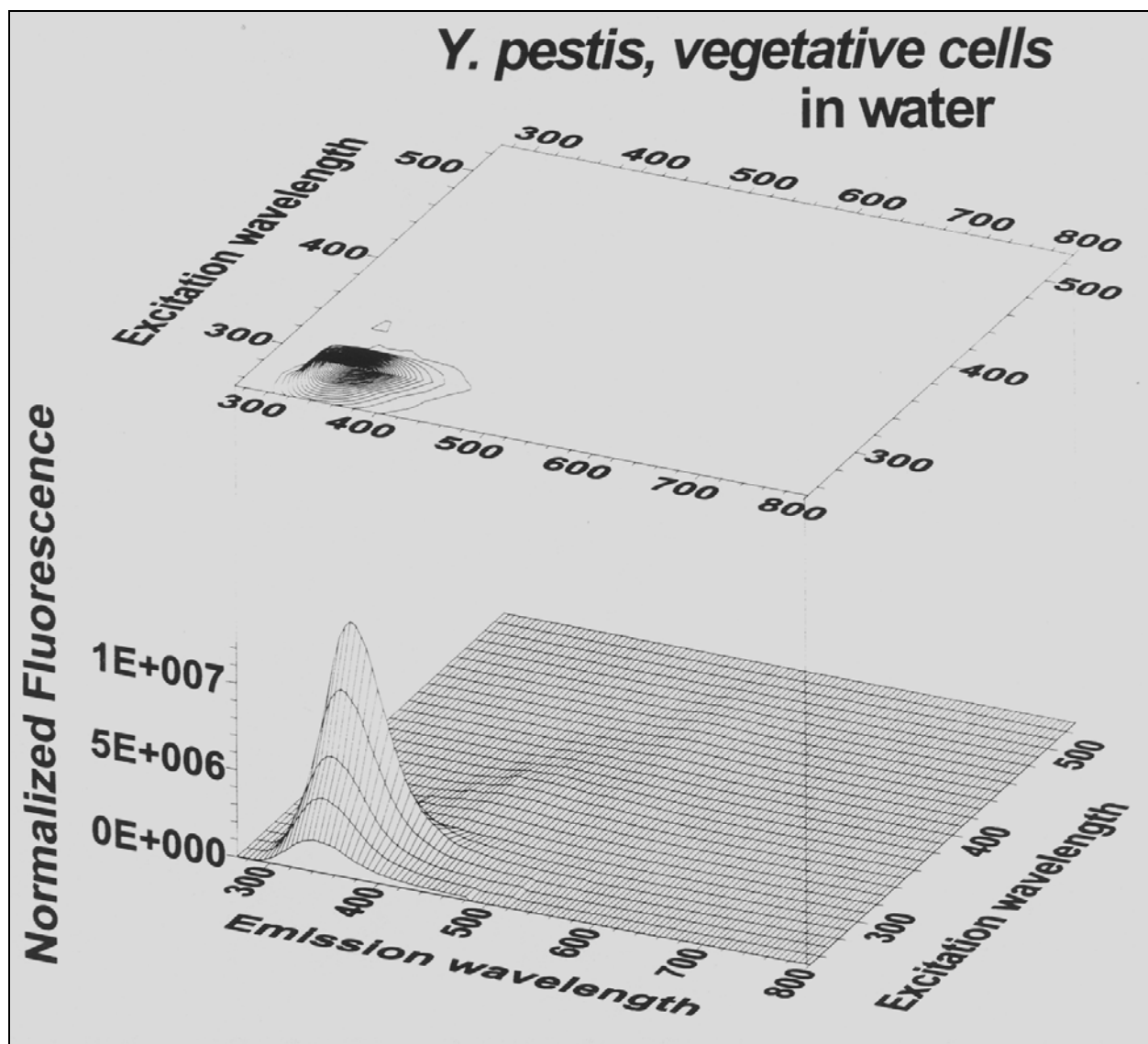


Figure 16. EEM spectra of *Yersinia pestis* vegetative cells, in water.

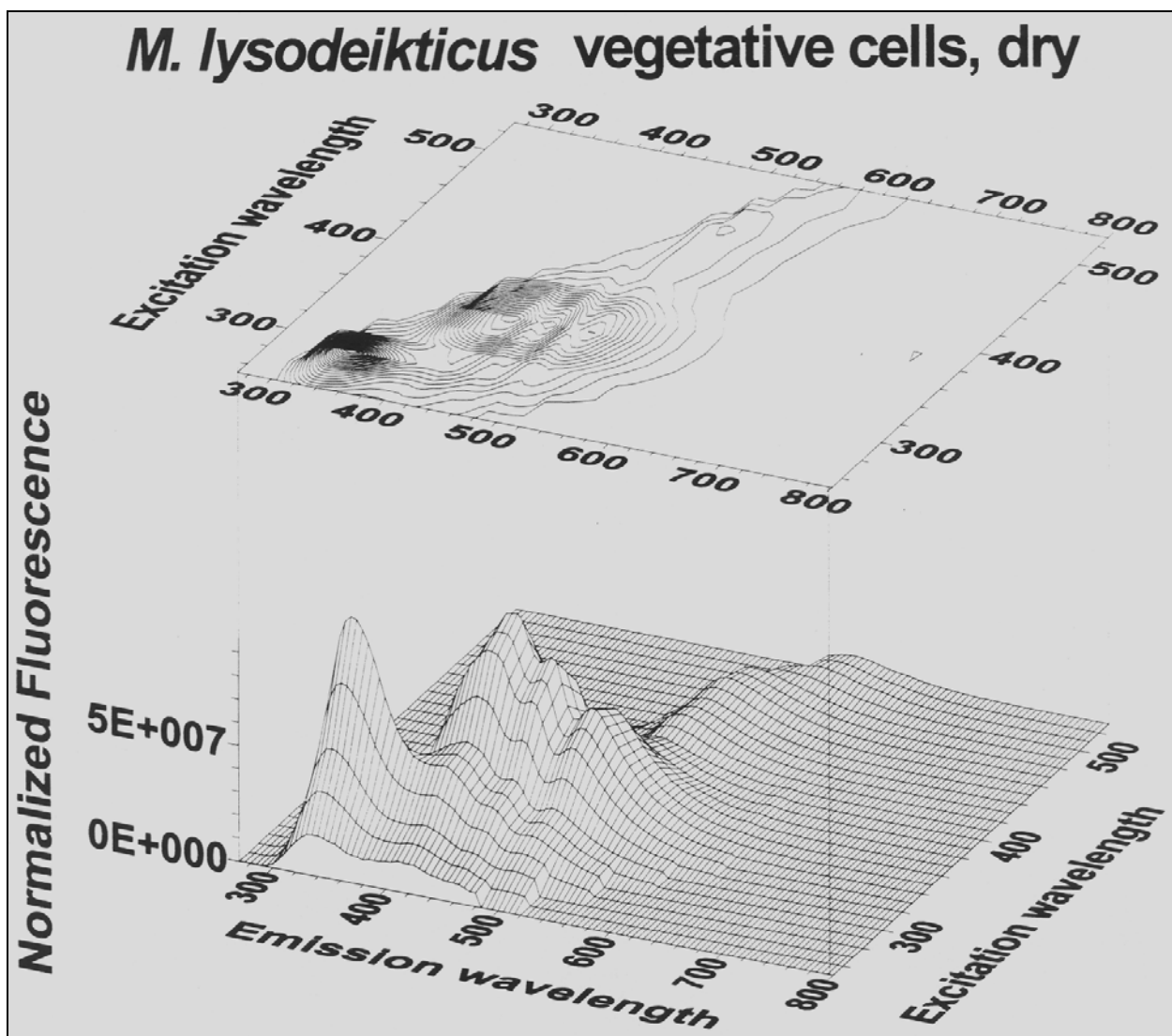


Figure 17. EEM spectra of *Micrococcus lysodeikticus* vegetative cells, dry.

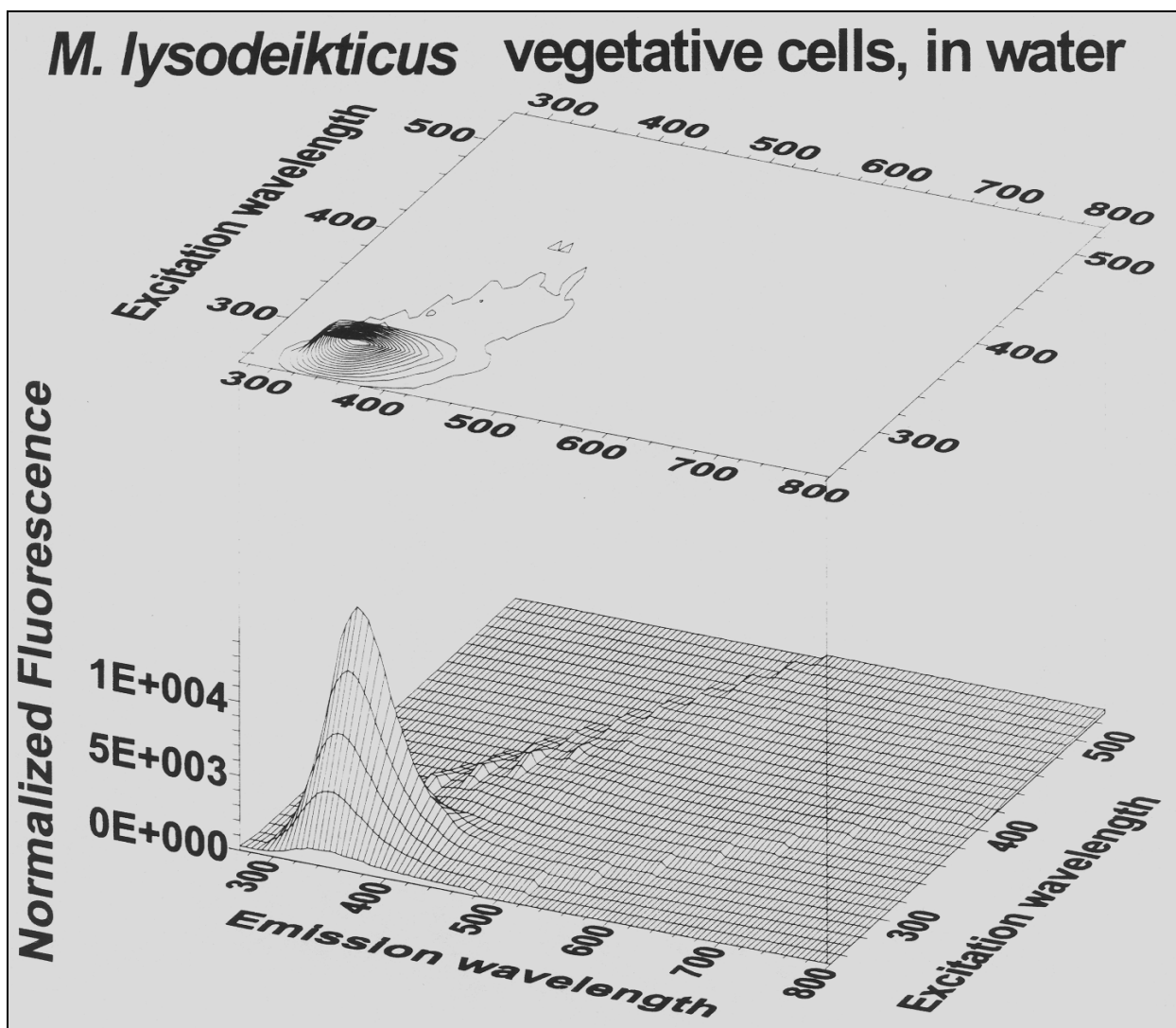


Figure 18. EEM spectra of *Micrococcus lysodeikticus* vegetative cells, in water.

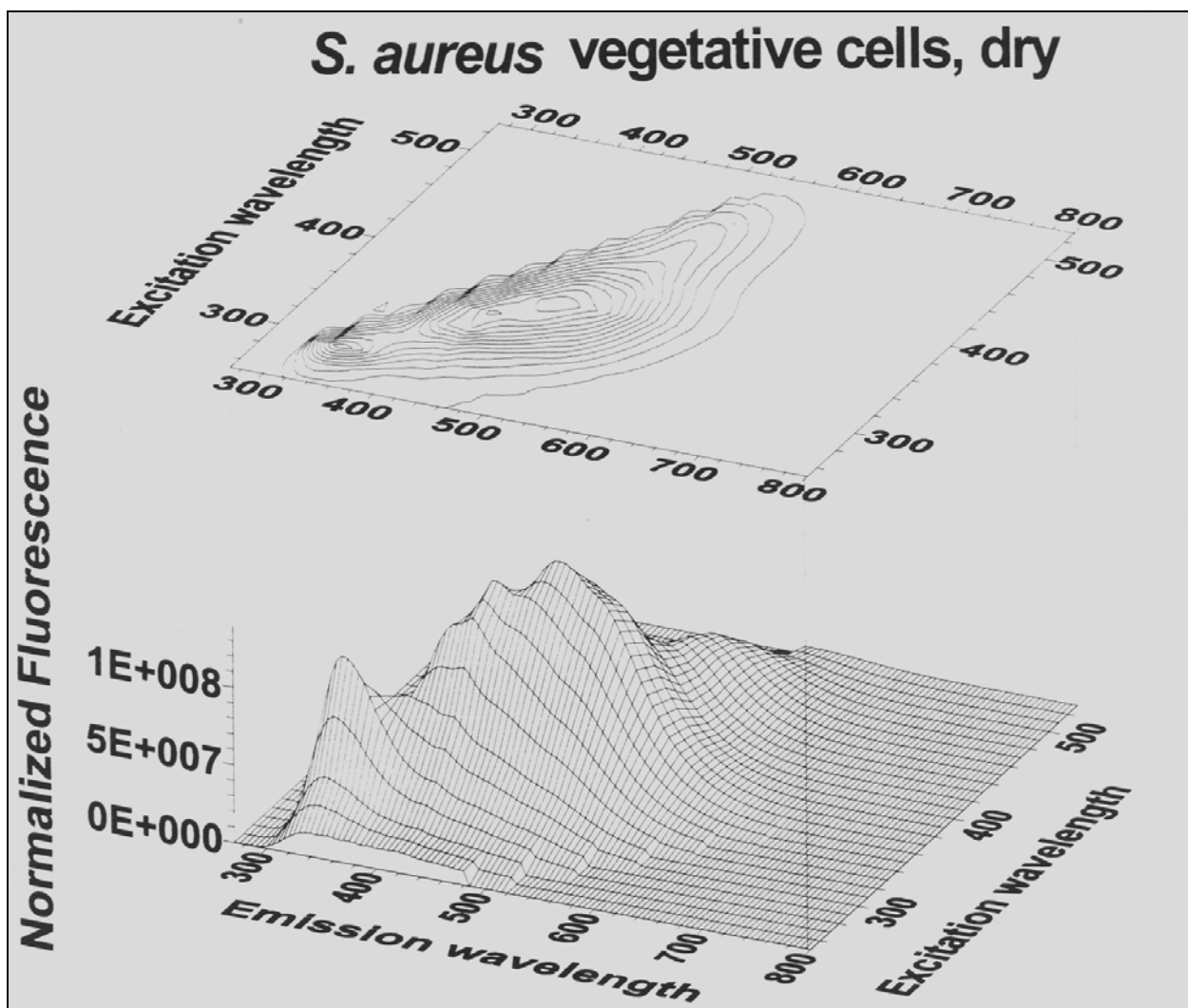


Figure 19. EEM spectra of *Staphylococcus aureus* vegetative cells, dry.



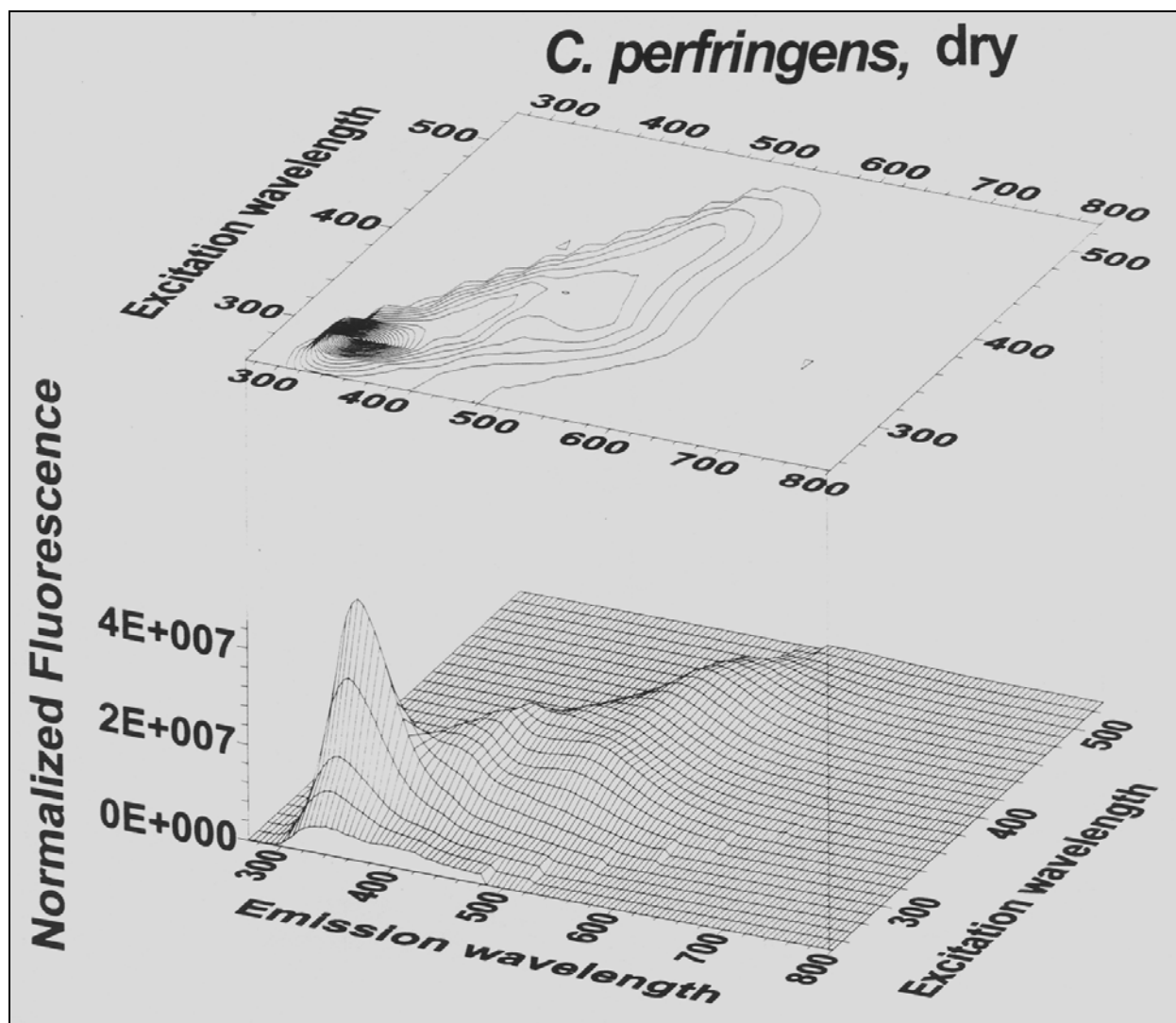


Figure 20. EEM spectra of *Clostridium perfringens* vegetative cells, dry.

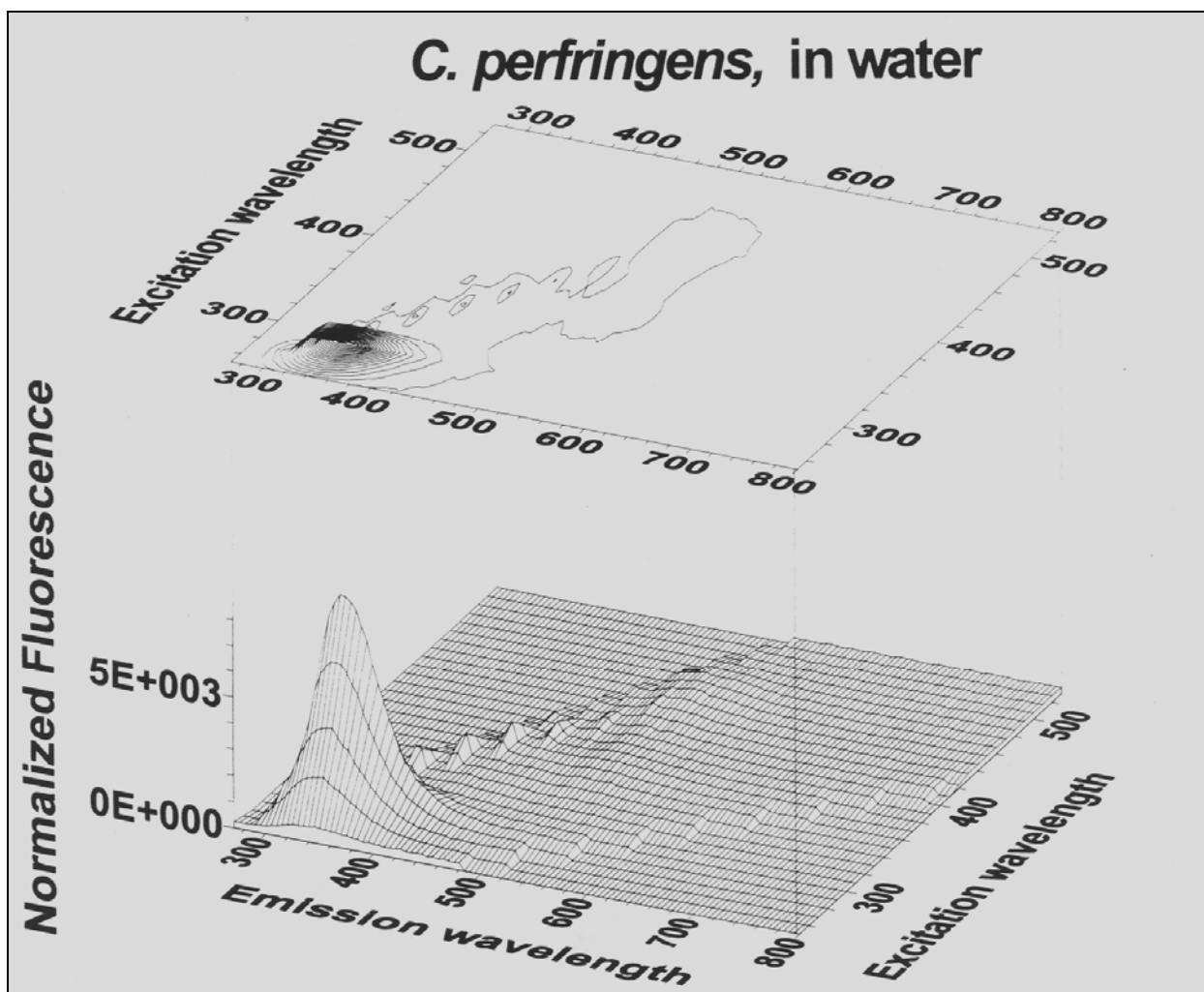


Figure 21. EEM spectra of *Clostridium perfringens* vegetative cells, in water.

### 3.3 Pollens and Fungal Spores

Figures 22–27 are EEM spectra of pollens (pecan, paper mulberry, corn, and ragweed), puff ball spores (fungal spores), and sporopollenin particles (from the spore coat of a club moss *Lycopodium*). These puff ball spores and the sporopollenin exhibited very low fluorescence. The fluorescence intensity from the pollens is more than an order of magnitude larger than that from the fungal spores.

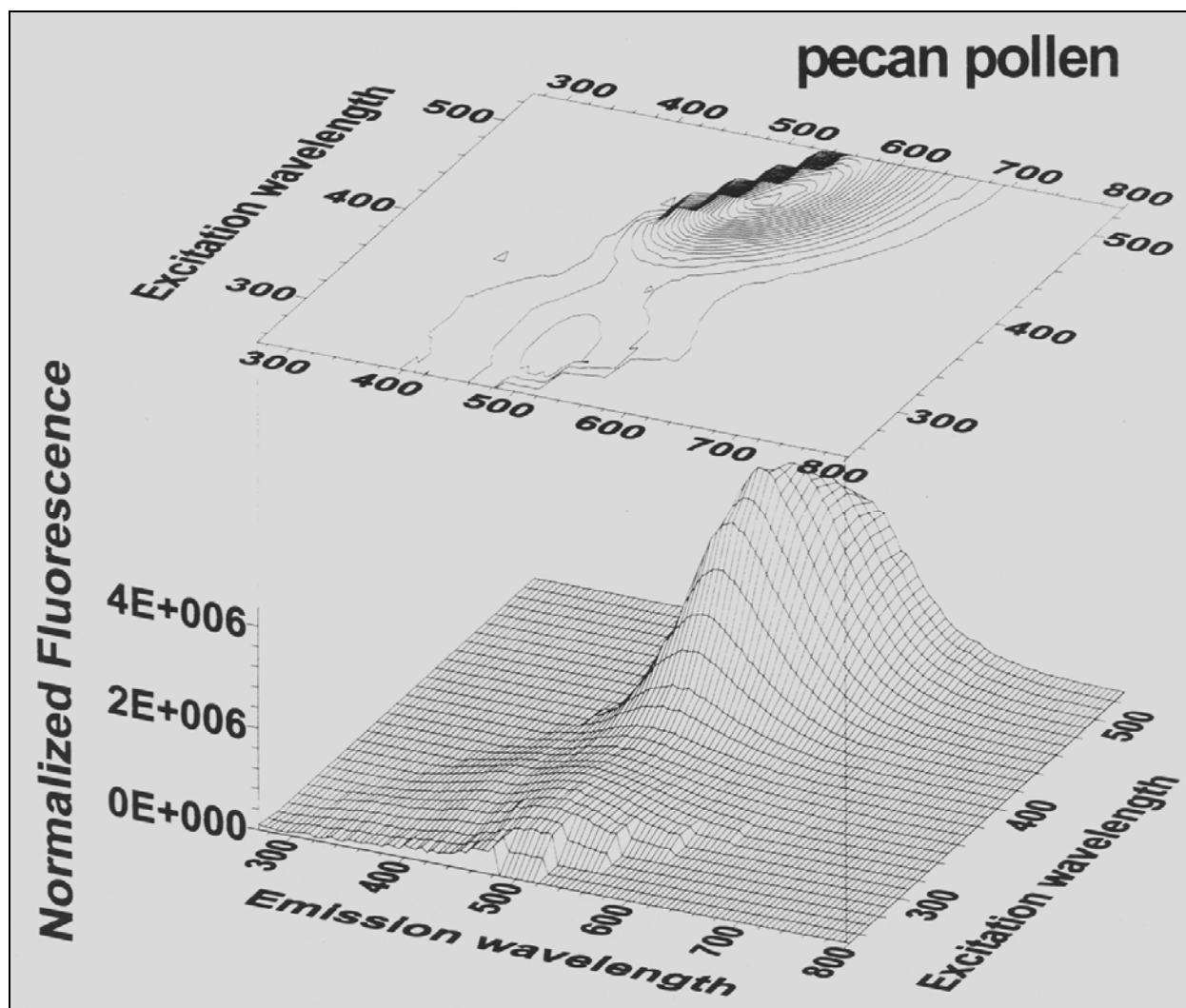


Figure 22. EEM spectra of pecan pollen, dry.

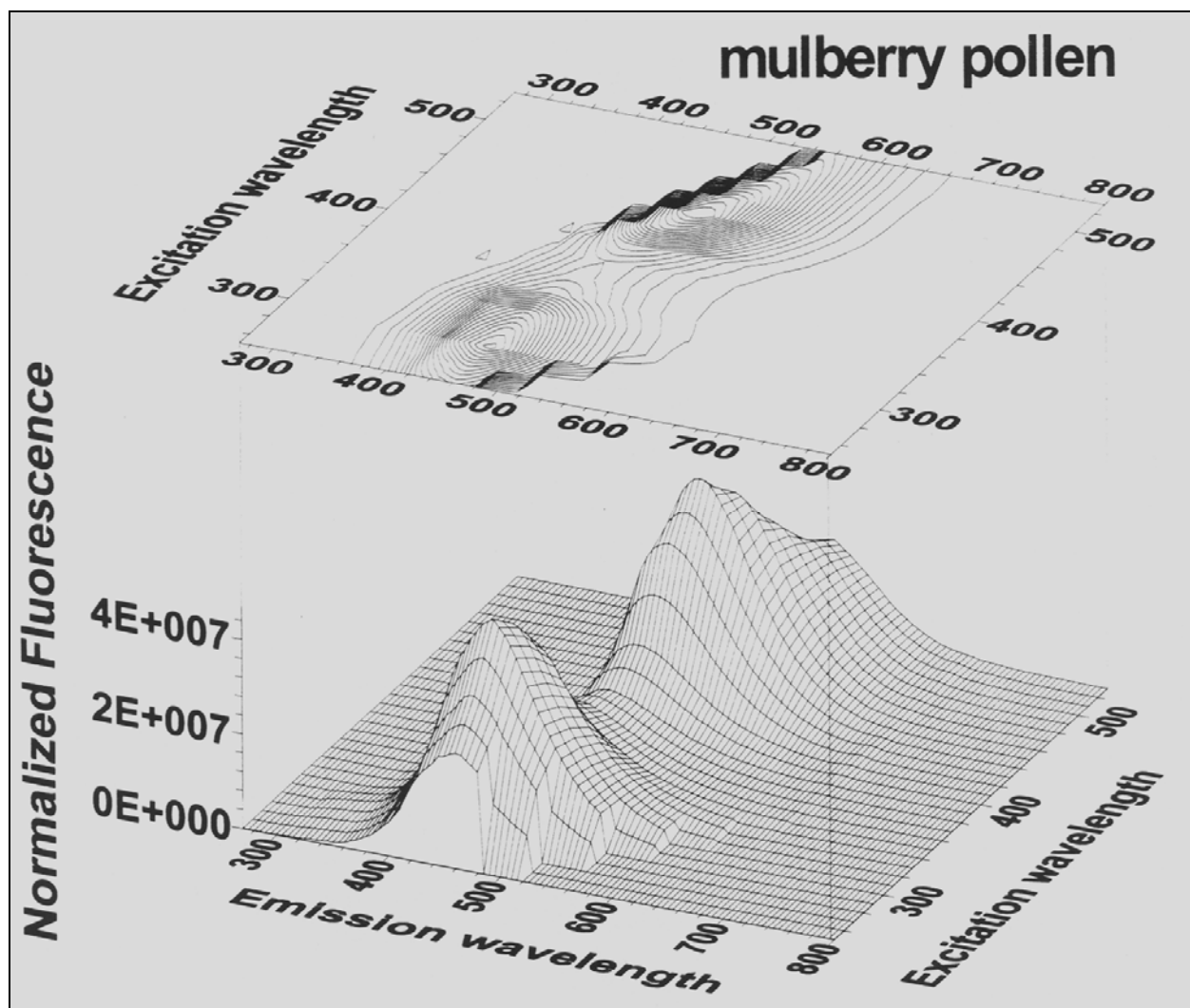


Figure 23. EEM spectra of paper mulberry pollen, dry.

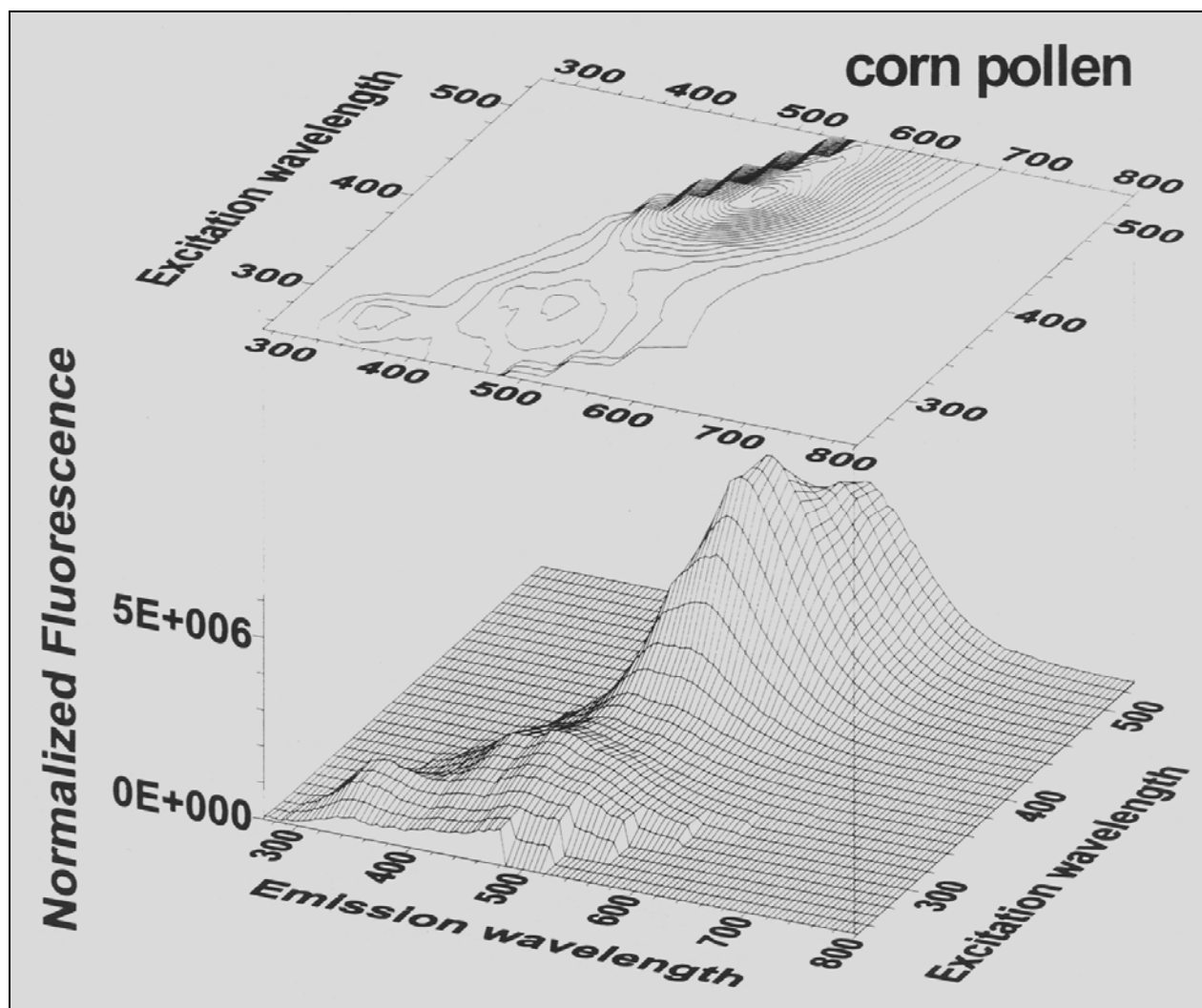


Figure 24. EEM spectra of corn pollen, dry.

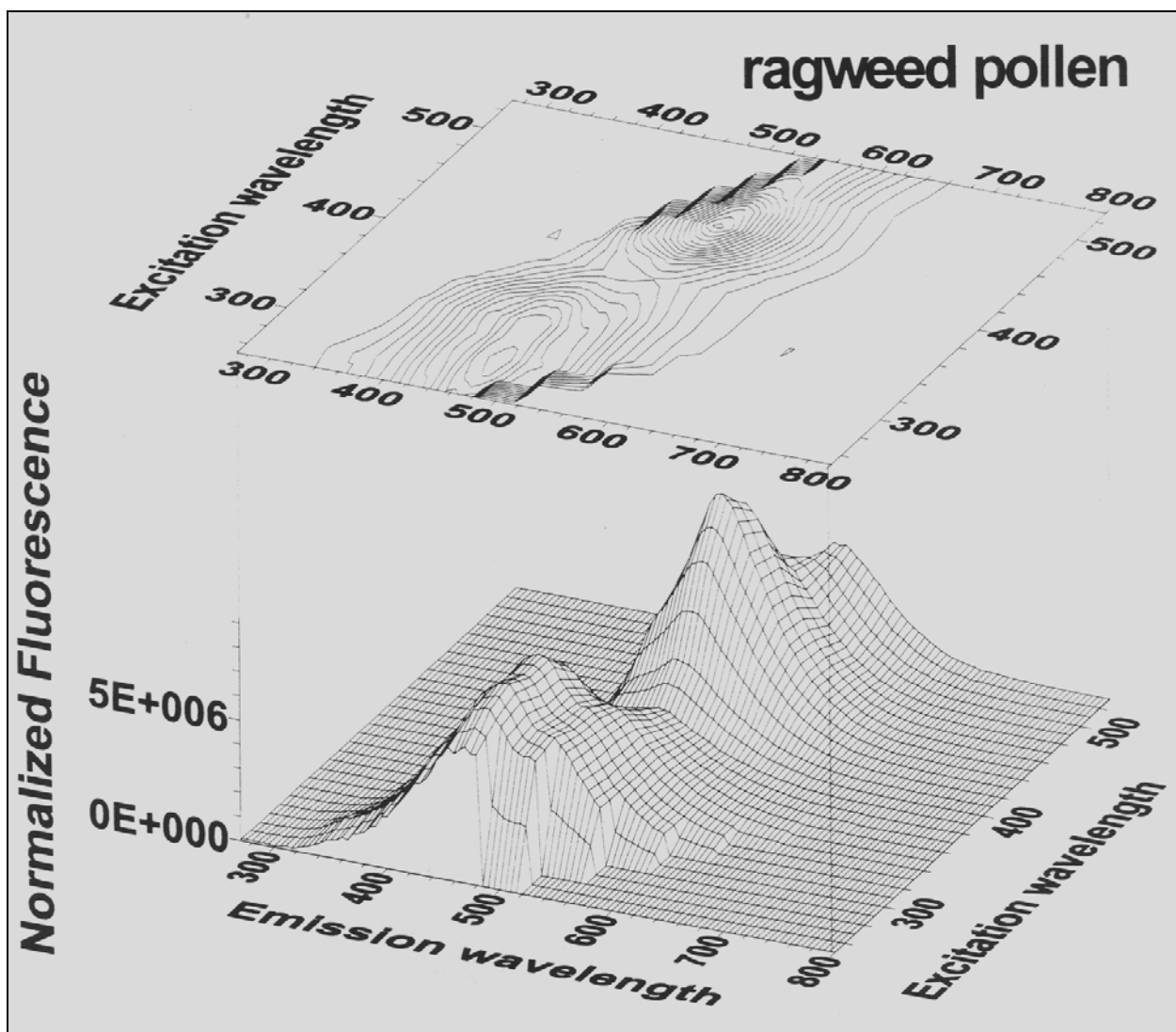


Figure 25. EEM spectra of ragweed pollen, dry.

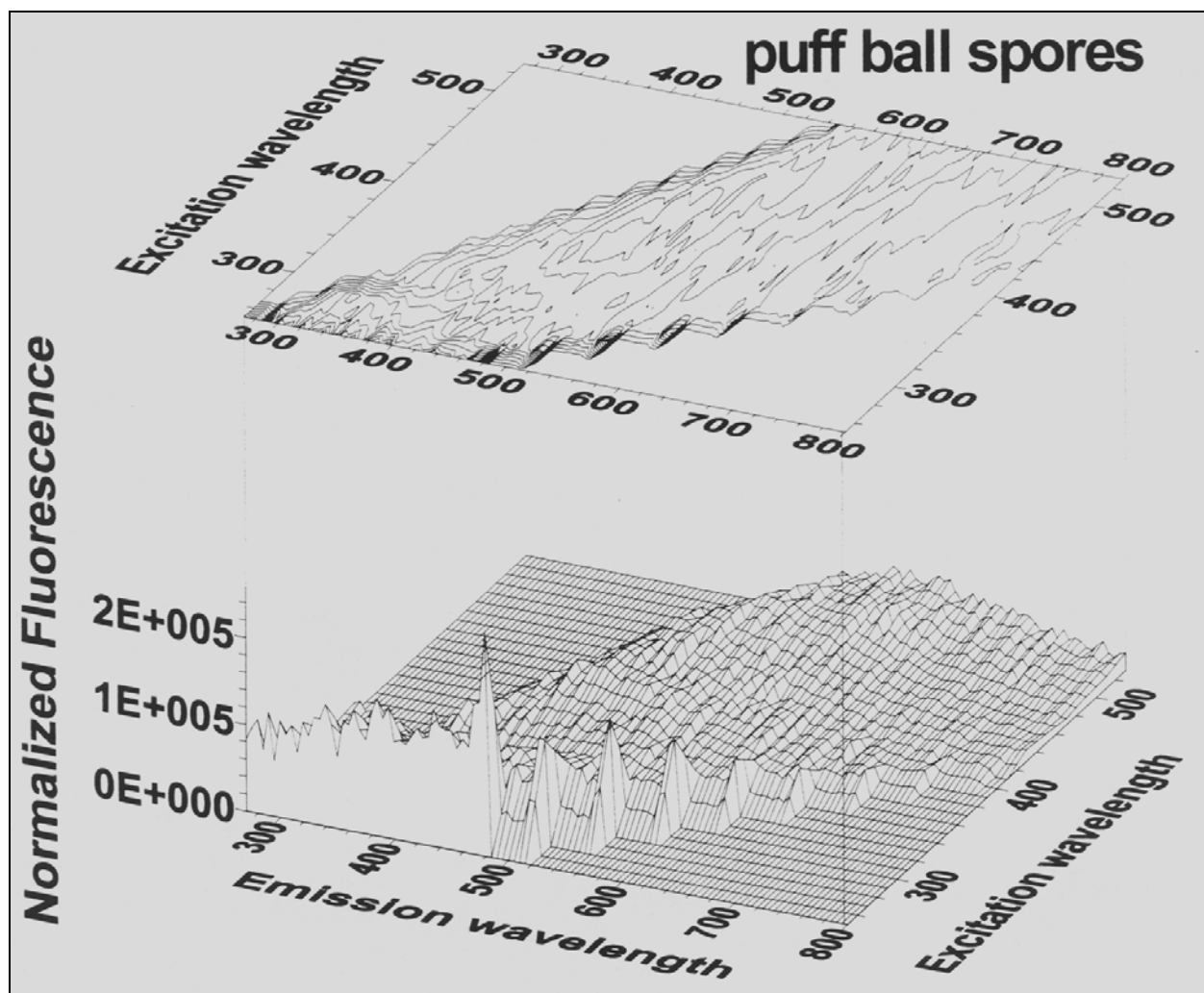


Figure 26. EEM spectra of puff ball spores, dry.

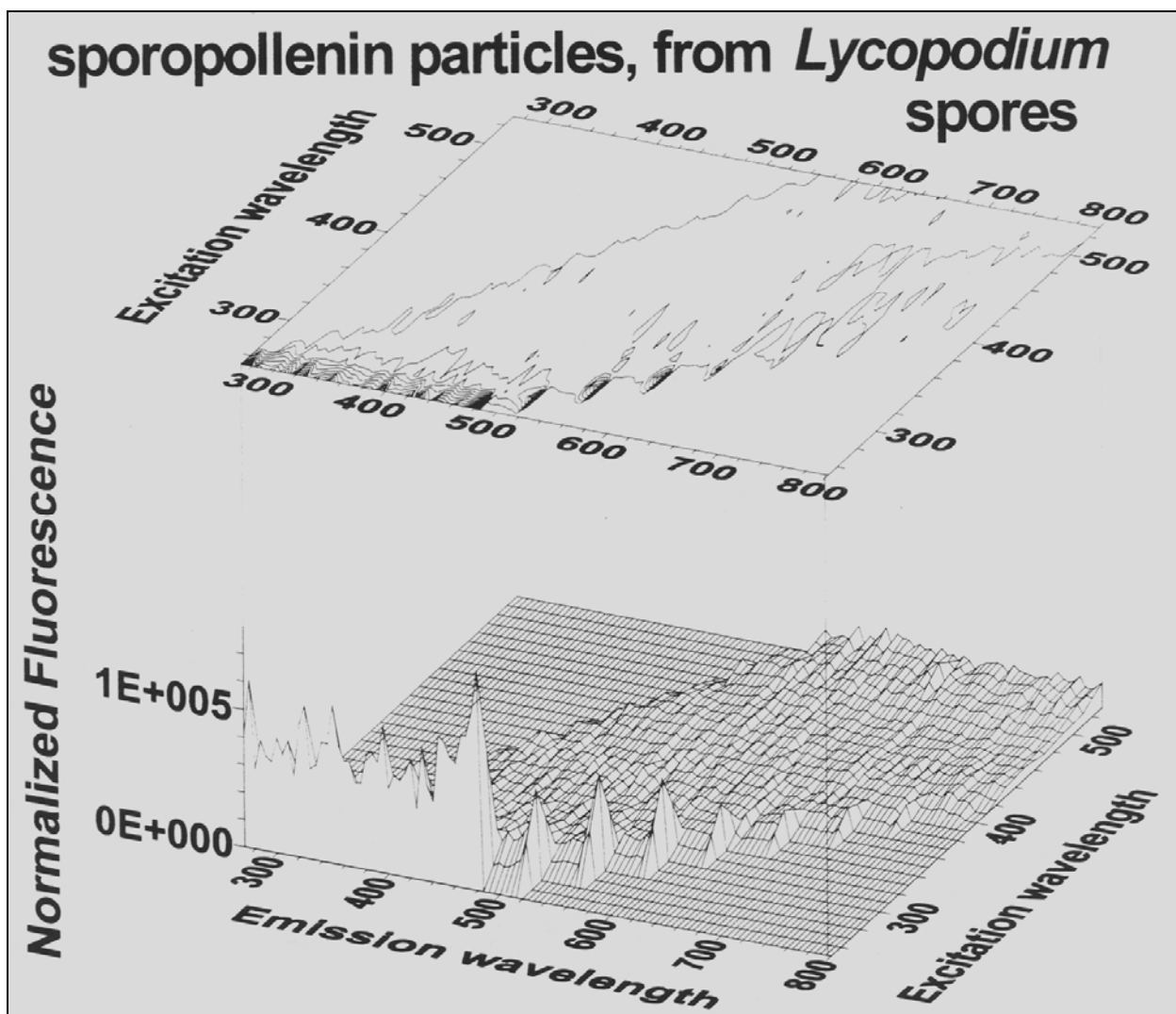


Figure 27. EEM spectra of sporopollenin from *Lycopodium* spores, dry.

### 3.4 Nonbiological Particles

Figures 28–31 show the EEM spectra of inorganic particles: glass beads and particles of kaolin, iron oxide (hematite), and metallic iron. The glass beads and kaolin particles have some weak fluorescence. As with some other weakly fluorescing compounds, sharp peaks (which are artifacts) occur near the second order of the excitation wavelength. The hematite and metallic iron particles have such a low fluorescence (and/or such large absorption) that their spectra appear to consist of nothing but the photomultiplier tube (PMT) noise and second-order excitation wavelength artifacts.



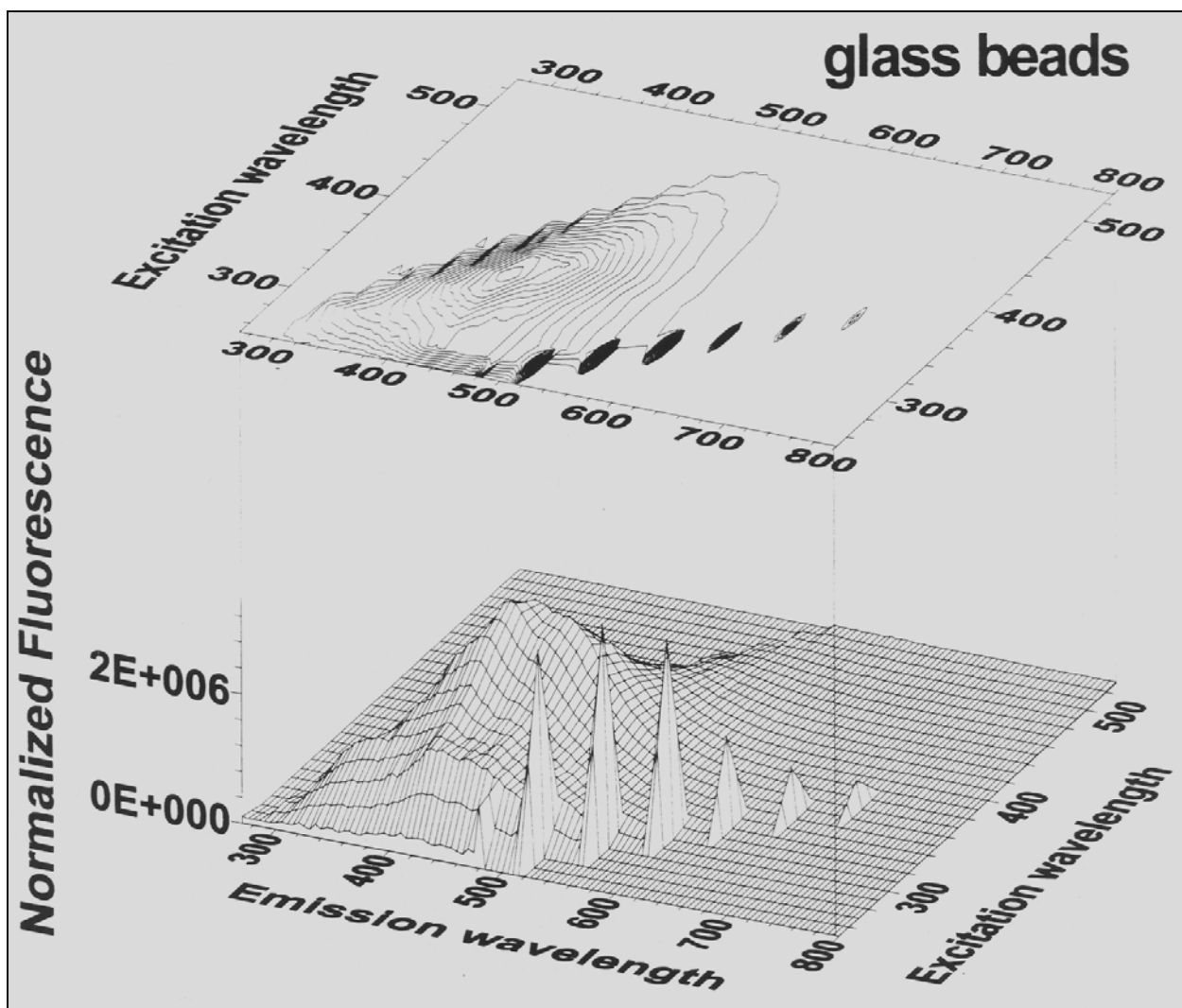


Figure 28. EEM spectra of glass beads, dry.

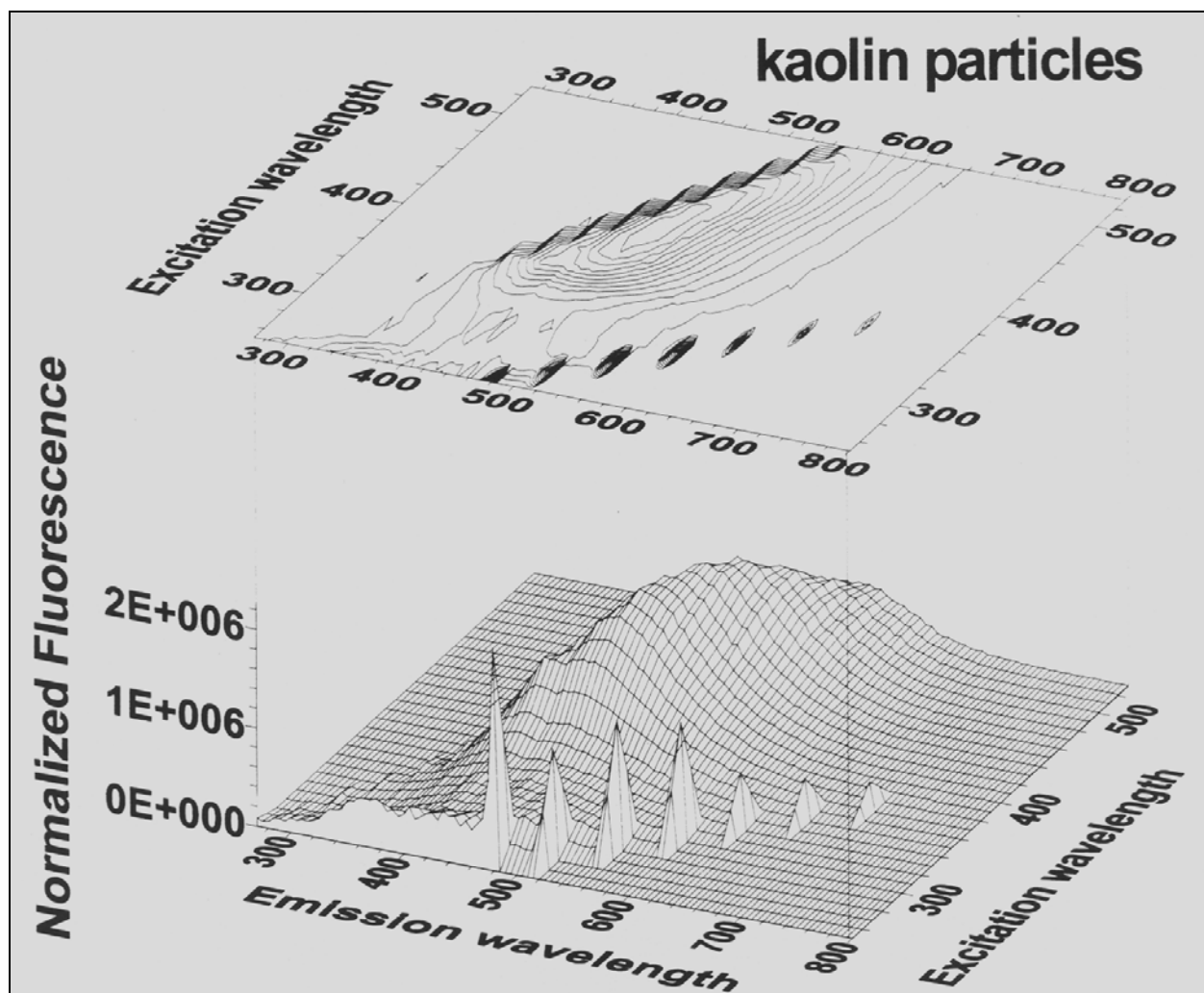


Figure 29. EEM spectra of kaolin particles, dry.

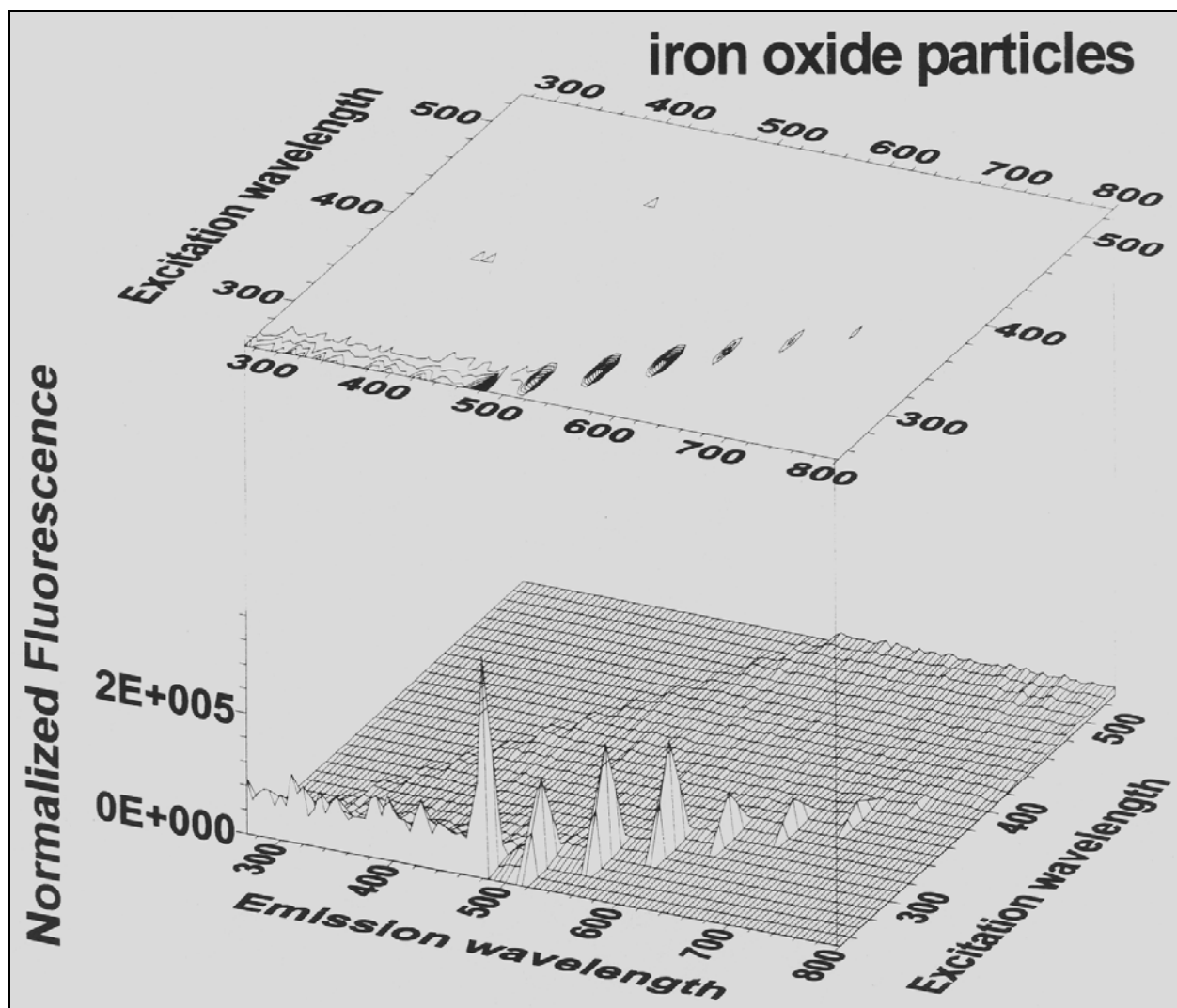


Figure 30. EEM spectra of iron oxide particles, dry.

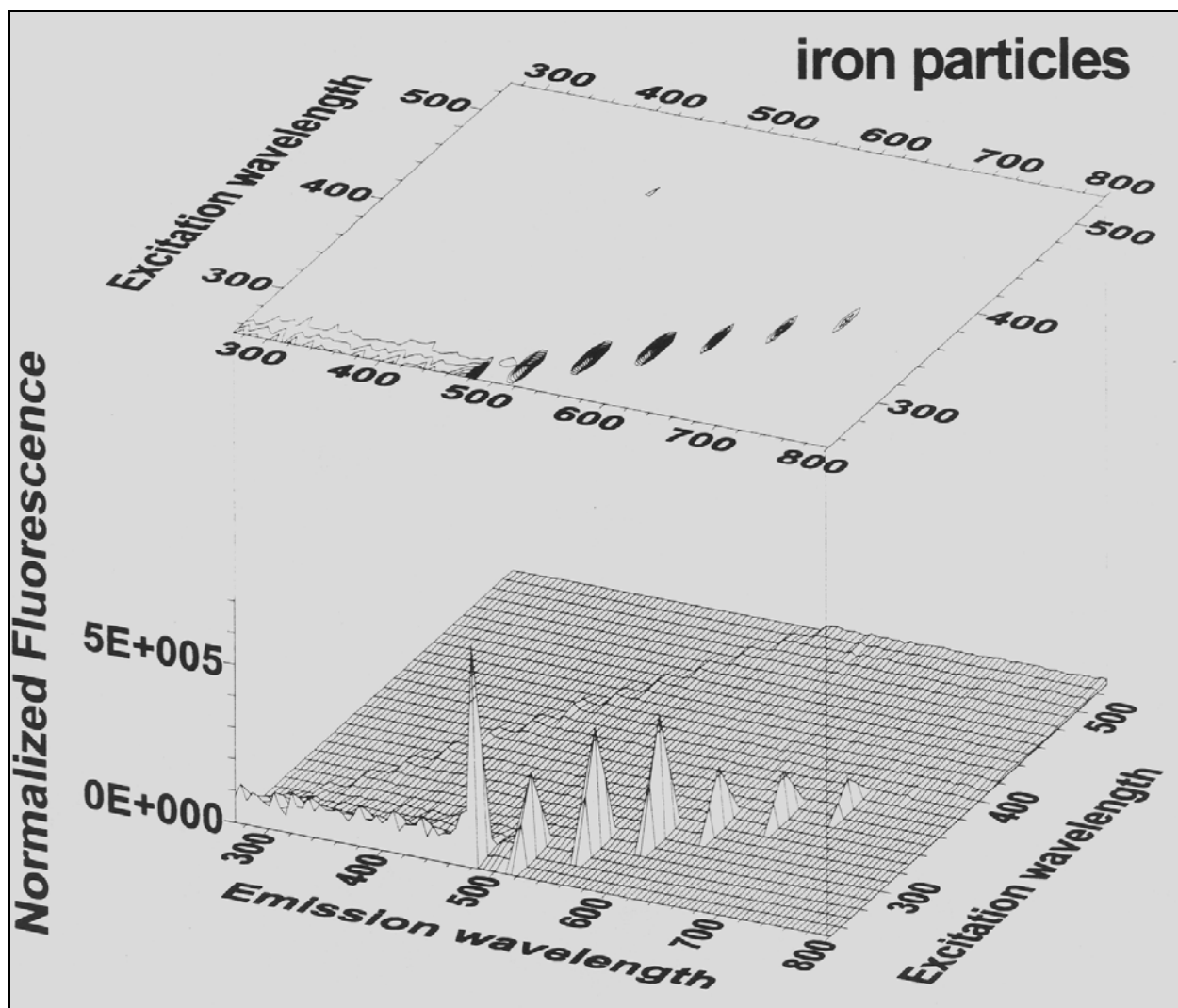


Figure 31. EEM spectra of iron particles, dry.

Figure 32 shows the spectra of an oil used for generating artificial fog; it exhibits a strong fluorescence.

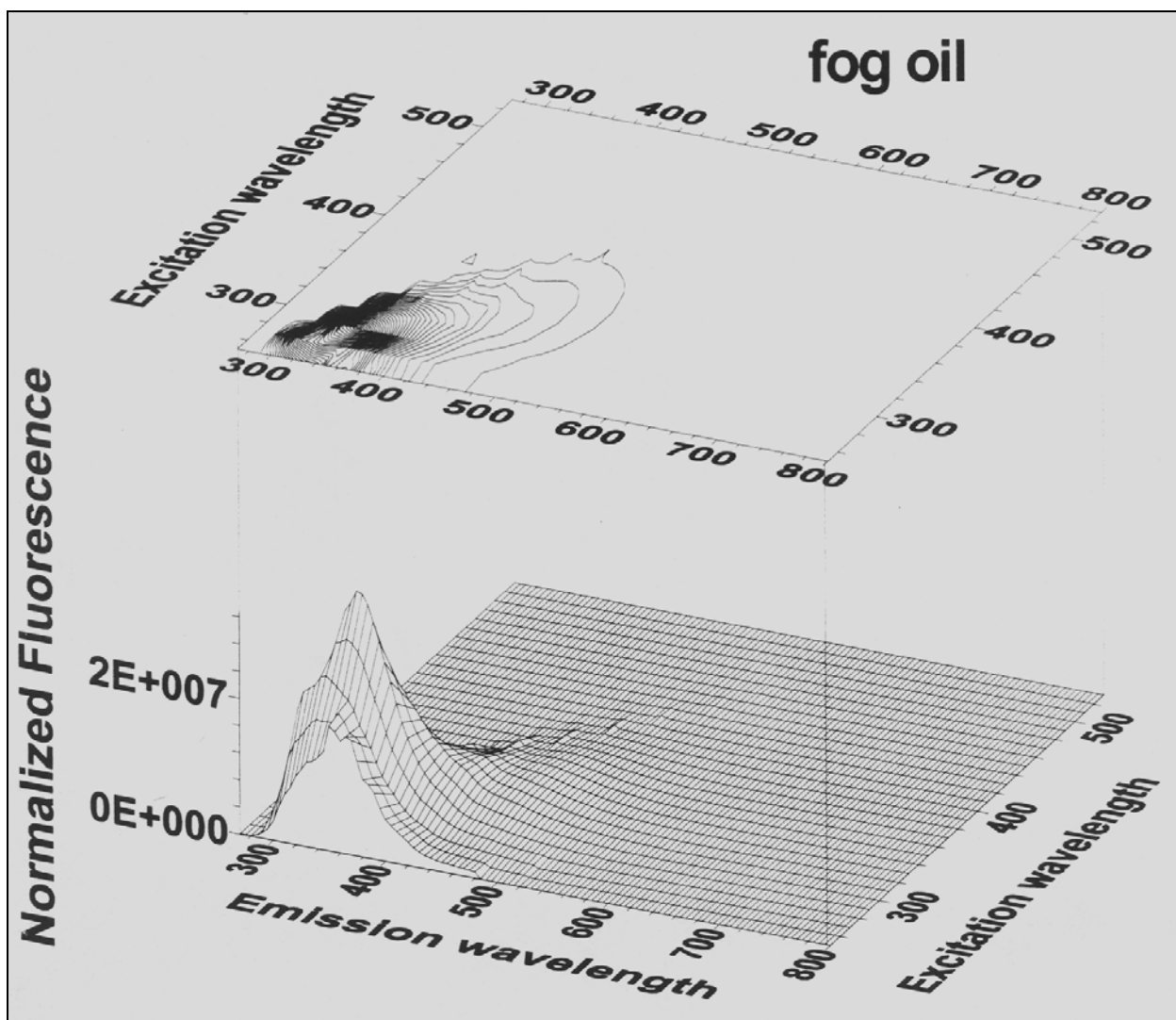


Figure 32. EEM spectra of fog oil.

#### 4. Discussion of EEM Spectra

The EEM data demonstrate that there are large variations in fluorescence between the samples shown. The spectra from biological samples are consistent with the assumption that flavins, NADH compounds, and the fluorescing amino acids (and chlorophylls in photosynthetic plants) are the major fluorescent materials in most of the cells and spores shown.

From the EEM spectra shown, it is not possible to state how much of the variation between biological samples is attributable to growth conditions or to differences in species. Some work on the variations in fluorescence of cells as a function of growth conditions has been reported (69, 57). More work is needed.

#### 4.1 Dry Versus Wet Samples

We have been particularly interested in dry samples because airborne bacteria, spores and pollens are often dry. However, bacteria (and less commonly spores and pollens) also occur in droplets, and so their spectra in water are also of interest, even for bioaerosols. The differences between the spectra of dry samples and samples in water can be striking. For example, riboflavin's peak emission has a large shift toward the red (from 520 to 565 nm) when going from a solution in water to a solid sample (see figure 4). We do not know the reason for this shift. However, one contributing factor may be as follows. Riboflavin can absorb energy at wavelengths larger than 530 nm. In the solid sample, the riboflavin is so concentrated that light emitted at shorter wavelengths may be reabsorbed and possibly re-emitted, while light at longer wavelengths is not absorbed as much and is able to exit from the sample.

The dry bacterial samples appear to have more fluorescence at longer wavelengths than do the wet samples (suspensions in water). Again, in the dry samples, the molecules are more concentrated and some of the fluorescence may be reabsorbed and may be partially re-emitted at longer wavelengths.

#### 4.2 Limitations of Applying EEM Spectra of Bulk Samples to Airborne Particles

EEM spectra of bulk samples can help in determining useful fluorescence excitation and emission wavelength ranges for discrimination among airborne biological and nonbiological particles. However, fluorescence from bulk samples, particularly the relative intensities of different samples, can be different from the fluorescence from airborne samples.

In bulk, if a material has a weak fluorescence, but also a weak absorption, the penetration depth in the material  $\delta_w$  may be large (centimeters), so that a large volume of the material may be excited by the incident light. The fluorescence detected is the fluorescence per volume multiplied by the volume sampled. In a weakly absorbing bulk sample, the fluorescence detected comes from a large volume of the sample. On the other hand, a highly fluorescent bulk sample, which is also highly absorbing of the incident light, has a much shorter penetration length  $\delta_s$  at the illumination wavelength (e.g., a few  $\mu\text{m}$ ). The light collected by the PMT comes from a volume that may be many times smaller (the ratio  $\delta_s/\delta_w$ ) than the sampled volume of the low-absorbing, low-fluorescing sample. Therefore, even though the highly fluorescent material is many times more fluorescent per volume, the detected fluorescence may be only a few times larger than from the sample with the weak fluorescence per volume.

In contrast, if the sample is a small particle, then even if the penetration depth in the material is essentially infinite, the sampled volume still is no larger than the volume of the particle. If the material has a weak fluorescence per volume, then the detected fluorescence will be very small. On the other hand, if the particle diameter is as small as a few  $\mu\text{m}$ , then even if the penetration depth is as small as few  $\mu\text{m}$ , a significant fraction of the total volume will be illuminated and

sampled. Then since the sampled volumes are comparable, the fluorescence from the highly fluorescent material will be many times stronger than the signal from the weakly fluorescing material.

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## **5. Discussion of Appropriate Wavelengths and Sources for Discrimination**

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The data shown are relevant to the detection of both airborne and non-airborne samples. Because our interest is primarily in bioaerosols, we limit our discussion to detection of aerosols. In developing improved autofluorescence-based bioaerosol sensing instruments, we want to determine the most useful sources for discriminating between biological and nonbiological particles, or among different types of biological particles. An appropriate excitation wavelength (1) should generate fluorescence that has spectra, which contain information for discrimination among types of biological particles and between biological and nonbiological particles; and (2) should not unnecessarily excite interfering fluorescence from nonbiological particles. To achieve (1), the excitation wavelength should be short enough that it excites different types of biological compounds. To achieve (2), it is best to use the longest wavelengths that excite the fluorescence of interest (i.e., from flavins, NADH, fluorescing amino acids, etc.).

### **5.1 Excitation Wavelengths for Discrimination Between Biological and Nonbiological Particles**

The EEM spectra shown here suggest that for discriminating between nonbiological particles and biological cells (including spores or pollens), continuous wave (cw) excitation sources, which can excite flavins (e.g., excitation below about 530 nm) or which can excite NADH and flavins (excitation below 420 nm), are appropriate. However, some samples, e.g., *B. subtilis* vegetative cells in water (figure 11) or *E. coli* in water (not shown), appear to exhibit little fluorescence beyond that expected from the fluorescing amino acids in proteins (e.g., the emission above 400 nm is very small if not negligible).

For detecting bioaerosols that may contain no flavins or NADH, (e.g., viruses composed of proteins and nucleic acids), excitation sources that excite protein fluorescence are required. Also, dilute suspensions of bacteria inside of water droplets probably also require excitation wavelengths below about 315 nm (see, e.g., figure 8b). The ratio of the protein fluorescence to the emissions at longer wavelengths can help in discriminating between biological and nonbiological particles.

### **5.2 Excitation Wavelengths for Discrimination Among Biological Particles**

For discrimination among biological particles, composed of, algae, bacterial spores, and/or pollens, excitation wavelengths that excite all four of the main types of fluorescing compounds (amino acids NADH compounds, flavins, and chlorophylls) may provide the most information.

Wavelengths above ~420 nm, for example, do not excite NADH or amino acids and will provide less information. On the other hand, in measurements of single-particle LIF from atmospheric particles using 263-nm excitation, we have not measured fluorescence we could attribute to chlorophyll. The EEM spectra demonstrate that excitation below about 315 nm can excite fluorescence in pure tryptophan. Again, as the excitation wavelength decreases, more nonbiological molecules are excited and can interfere with the detection of the bioaerosols. The EEM spectra shown here suggest that for discriminating among biological particles it may be useful to use multiple excitation wavelengths. Measurements of atmospheric particles with multiple excitation wavelengths also appear to be useful (37, 44).

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## **6. Conclusions**

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EEM spectra should be useful for designing autofluorescence-based instruments for discriminating between biological and nonbiological airborne particles, and among limited numbers of possible types of biological particles. In the measurements shown here, fluorescence in the regions where NADH and flavins emit was detected from a variety of bacterial cells and spores, particularly in the dry samples. The fluorescence of the dry samples appears to be shifted to longer wavelengths, possibly because of re-absorption of light and subsequent re-emission. Fluorescence spectra in themselves are inadequate for accurate species determinations: specific identification of bacteria and viruses requires specific antibody or nucleic acid probes for each type of biological particle of interest. Nevertheless, autofluorescence-based bioaerosol detectors should be useful for prescreening, and for indicating when it might be appropriate to turn on more specific identifiers. Also, the relative nonspecificity of an autofluorescence-based bioaerosol detector can be an advantage in cases where there are many types of unknown bacteria, pollens, or biomolecules, since it requires no prior synthesis of specific probes.



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## 7. References

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## Acronyms

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EEM	excitation and emission
FAD	flavin adenine dinucleotide
FMN	flavin mono nucleotide
IR	infrared
LIBS	Laser-induced breakdown spectroscopy
LIF	laser-induced fluorescence
LPCs	laser particle counters
NADH	nicotinamide-adenine dinucleotide
OCA	organic carbon aerosols
PMT	photomultiplier tube
UV	ultraviolet

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